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# Protocols for Short Term Toxicity Screening of Hazardous Waste Sites

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# PROTOCOLS FOR SHORT TERM TOXICITY SCREENING OF HAZARDOUS WASTE SITES

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This manual contains short-term methods for measuring the toxicity of chemical contaminants in soil, sediment, surface water, and groundwater samples. The algal assay is a chronic test, while all other tests described in the manual are acute tests. The methods are one of several tools, including chemical analysis and field study, used to determine toxicity of hazardous waste sites. The toxicity tests provided in this manual can be used to detect toxic materials, rank sites based on relative short-term toxicity, and provide a cost-effective approach to monitoring the effectiveness of site cleanup.

The toxicity tests in this manual are not required by regulation. However, because toxicity tests measure the integrated effects of complex chemical waste mixtures, they provide a reasonable basis for assessing the toxicity of waste products independent of existing concentration criteria.

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## EXECUTIVE SUMMARY

This manual contains short-term methods for measuring the toxicity of chemical contaminants in soil, sediment, surface water, and groundwater samples. The algal assay is a chronic test, while all other tests described in the manual are acute tests. The methods are one of several tools, including chemical analysis and field study, used to determine toxicity of hazardous waste sites. The toxicity tests provided in this manual can be used to detect toxic materials, rank sites based on relative short-term toxicity, and provide a cost-effective approach to monitoring the effectiveness of site cleanup.

The methods include aquatic and terrestrial tests. The algae, macroinvertebrate, and root elongation tests are used to assess toxicity in surface water, groundwater, and sediment or soil elutriates. The earthworm and seed germination tests are used to directly assess toxicity in soil and sediment samples.

A critical factor in waste site evaluation is establishment of an experimental design that satisfies the information needs for site evaluation. Components of experimental design (sampling, statistical considerations, selection of biological tests, logistical support requirements, etc.) are discussed in the companion document entitled, **"Role of Acute Toxicity Bioassays in the Remedial Action Process at Hazardous Waste Sites"** (Athey et al. 1987).

The toxicity tests in this manual are not required by regulation. However, because toxicity tests measure the integrated effects of complex chemical waste mixtures, they provide a reasonable basis for assessing the toxicity of waste products independent of existing concentration criteria.

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## GLOSSARY

**Acclimation**--(1) Steady state compensatory adjustments by an organism to the alteration of environmental conditions. Adjustments can be behavioral, physiological, or biochemical. (2) Referring to the time period prior to the initiation of a toxicity test in which organisms are maintained in untreated, toxicant-free dilution water or soil with physical and chemical characteristics, e.g., temperature, pH, hardness, similar to those to be used during the toxicity test.

**Acute**--Involving a stimulus severe enough to rapidly induce a response. An acute effect is not always measured in terms of lethality; it can be measured in terms of a variety of endpoints. Note that acute means short, not mortality.

**Additivity**--The characteristic property of a mixture of toxicants that exhibits a cumulative toxic effect equal to the arithmetic sum of the effects of the individual toxicants.

**Alkalinity**--The acid-neutralizing (proton-accepting) capacity of water; the quality and quantity of constituents in water which result in a shift in the pH toward the alkaline side of neutrality.

**Antagonism**--The characteristic property of a mixture of toxicants that exhibits a less-than-additive cumulative toxic effect.

**Artificial soil**--Non-toxic synthetic soil, prepared in the laboratory using a specific formulation, used as a control and diluent medium in the earthworm and lettuce seed germination tests.

**Biological oxygen demand (BOD)**--The amount of oxygen necessary for the oxidative decomposition of a material by microorganisms.

**Biota**--Animal and plant life.

**Carcinogenic**--Capable of causing cancer.

**Chronic**--Involving a stimulus that lingers or continues for a relatively long period of time, often one-tenth of the life span or more. Chronic should be considered a relative term depending on the life span of an organism. A chronic effect can be lethality, altered growth, reduced reproduction, etc.

**Clitellum**--Thickened saddle-like portion of certain mid-body segments in many oligochaetes and leeches; in some species it is prominent only in sexually mature individuals; forms rings of epithelial tissue and mucus as cocoons in which eggs are enclosed; during copulation it also gives off mucus which envelopes the anterior ends of the two individuals.

**Control**--A treatment in a toxicity test that duplicates all the conditions of the exposure treatments but contains no test material. The control is used to determine the absence of toxicity of basic test conditions, e.g., health of test organisms, quality of dilution water.

**Criteria (water quality)**--An estimate of the concentration of a chemical or other constituent in water which if not exceeded, will protect an organism, an organismal community, or a prescribed water use or quality with an adequate degree of safety.

**Dilution water**--Water used to dilute the test material in an aquatic toxicity test in order to prepare either different concentrations of a test chemical or different percentages of an aqueous sample for the various test treatments. The water (negative) control in a test is prepared with dilution water only.

**Effluent**--A complex waste material, e.g., liquid industrial discharge or sewage, which is discharged into the environment.

**Elutriate**--Solution obtained after adding water to the solid waste, shaking, and centrifuging (see Section A.7.5 in the Appendix).

**Ephippia**--Special postero-dorsal part of the carapace of certain female cladocera which contains one to several eggs usually fertilized.

**Exposure**--The interaction between a chemical or physical agent and a biological system.

**Hardness**--The concentration of ions in the water which will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water and is frequently expressed as mg/L calcium carbonate equivalent.

**Hazard**--Likelihood that a chemical will cause an injury or adverse effect under conditions of its production, use, or disposal.

**Hazardous substance**--A material that can pose a hazard causing deleterious effects in plants or animals. (A hazardous substance does not in itself present a risk unless an exposure potential exists.)

**Instar**--(1) Period or stage between molts of an insect during larval or nymph portion of the life history; instars are usually numbered, the first larval instar being that stage hatching from the egg and extending to the first ecdysis. (2) In the broad sense, the period between any two successive molts in an arthropod, nematode, tardigrade, etc.

**Leachate**--Water plus solutes that has percolated through a column of soil or waste.

**Lethal**--Causing death by direct action. Death of organisms is the cessation of all visible signs of biological activity.

**Loading**--Ratio of the animal biomass to the volume of test solution in an exposure chamber.

**Macroinvertebrates**--Large invertebrate organisms, sometimes arbitrarily defined as those retained by sieves with 0.425-mm to 1.0-mm mesh screens.

**Median effective concentration (EC50)**--The concentration of material to which test organisms are exposed that is estimated to be effective in producing some sublethal response in 50% of the test organisms. The EC50 is usually expressed as a time-dependent value, e.g., 24-h or 96-h EC50. The sublethal response elicited from the test organisms as a result of exposure to the test material must be clearly defined. For example, test organisms may be immobilized, lose equilibrium, or undergo physiological or behavioral changes.

**Median lethal concentration (LC50)**--The concentration of material to which test organisms are exposed that is estimated to be lethal to 50% of the test organisms. The LC50 is usually expressed as a time-dependent value, e.g., 24-h or 96-h LC50; the concentration estimated to be lethal to 50% of the test organisms after 24 or 96 h of exposure. The LC50 may be derived by observation (50% of the test organisms may be observed to be dead in one test material concentration), by interpolation (mortality of more than 50% of the test organisms occurred at one test concentration and mortality of fewer than 50% of the test organisms died at a lower test concentration; the LC50 is estimated by interpolation between these two data points), or by calculation (the LC50 is statistically derived by analysis of mortality data from all test concentrations).

**Monitoring test**--A test designed to be applied on a routine basis, with some degree of control, to ensure that the quality of water, elutriate, or hazardous waste has not exceeded some prescribed range. In a biomonitoring test, organisms are used as "sensors" to detect changes in the quality of water, elutriate, or hazardous waste. A monitoring test implies generation of information, on a continuous or other regular basis.

**Neonates**--Newly born or newly hatched individuals.

**Parts per billion (ppb)**--One unit of chemical (usually expressed as mass) per 1,000,000,000 ($10^9$) units of the medium (e.g., water) or organism (e.g., tissue) in which it is contained. For water, the ratio commonly used is micrograms of chemical per liter of water, 1 ug = 1 ppb; for tissues, 1 ug/kg = 1 ng/g = 1 ppb.

**Parts per million (ppm)**--One unit of chemical (usually expressed as mass) per 1,000,000 ($10^6$) units of the medium (e.g., water) or organism (e.g., tissue) in which it is contained. For water, the ratio commonly used is milligrams of chemical per liter of water, 1 mg = 1 ppm; for tissues, 1 mg/kg = 1 ug/g = 1 ppm.

**Parts per thousand (ppt)**--One unit of chemical (usually expressed as mass) per 1000 ($10^3$) units of the medium (e.g., water) or organism (e.g., tissue) in which it is contained. For water, the ratio commonly used is grams of chemical per liter of water, 1 g/L = 1 ppt; for tissues, 1 g/kg = 1 ppt. This ratio is also used to express the salinity of seawater, where the grams of chloride per liter of water is denoted by the symbol ppt. Full-strength seawater is approximately 35 ppt.

**Parts per trillion (pptr)**--One unit of material (usually expressed as mass) per 1,000,000,000,000 ($10^{12}$) units of the medium (e.g., water) or organism (e.g., tissue) in which it is contained. The ratio commonly used is nanograms of chemical per liter of water, 1 ng/L = 1 pptr; for tissues, 1 ng/kg = 1 pptr.

**Percentage (%)**--One unit of material (usually a liquid) per 100 units of dilution water. In tests with industrial wastes (effluents), test concentrations are normally prepared on a volume-to-volume basis and expressed as percent of material.

**Persistence**--That property of a toxicant that is a measurement of the duration of its effect. A persistent toxicant maintains its effect after mixing, degrading slowly. A non-persistent toxicant may have a quickly reduced effect as processes such as biodegradation, volatilization, photolysis, etc., transform the chemical or reduce its concentration.

**Phytotoxic**--Toxic to plants.

**Probability**--A number expressing the likelihood of occurrence of a specific event, such as the ratio of the number of outcomes that will produce a given event to the total number of possible outcomes.

**Quality assurance (QA)**--A program organized and designed to provide accurate and precise results. Included are selection of proper technical methods, tests, or laboratory procedures; sample collection and preservation; selection of limits; evaluation of data; quality control; and qualifications and training of personnel.

**Quality control (QC)**--Specific actions required to provide information for the quality assurance program. Included are standardizations, calibration, replicates, and control and check samples suitable for statistical estimates of confidence of the data.

**Standard sample**--A sample of constant and defined composition (e.g., synthetic water or artificial soil).

**Static**--Describing toxicity tests in which test materials are not renewed.

**Statistically significant effects**--Effects (responses) in the exposed population that are different from those in the controls at a prespecified probability level. Biological endpoints that are important for the survival, growth, behavior, and perpetuation of a species are selected as endpoints for effect. The endpoints differ depending on the type of toxicity test conducted and the species used. The statistical approach also changes with the type of toxicity test conducted.

**Sublethal**--Involving a stimulus below the level that causes death.

**Survival time**--The time interval between initial exposure of an organism to a harmful chemical and death.

**Synergism**--The characteristic property of a mixture of toxicants that exhibits a greater-than-additive cumulative toxic effect.

**Test material**--A chemical, formulation, elutriate, sludge, or other agent or substance that is under investigation in a toxicity test.

**Test soil**--A mixture of contaminated site soil and artificial soil to provide a medium for exposing test organisms to solid hazardous waste.

**Test solution (or test treatment)**--Medium containing the material to be tested to which the test organisms are exposed. Different test solutions contain different concentrations of the test material.

**Toxicant**--An agent or material capable of producing an adverse response (effect) in a biological system, adversely impacting structure or function or producing death.

**Toxic endpoints**--Measurements of acute or chronic toxicity for toxic substances, including exposure duration, concentration, and observed effects.

**Toxicity**--The inherent potential or capacity of a material to cause adverse effects in a living organism.

**Toxicity curve**--The curve obtained by plotting the median survival times of a population of test organisms against concentration on a logarithmic scale.

**Toxicity test**--The means by which the toxicity of a chemical or other test material is determined. A toxicity test is used to measure the degree of response produced by exposure to a specific level of stimulus or concentration of a chemical.

**Water holding capacity**--The quantity of water that soil retains after 24 hours following complete saturation, generally expressed as ml/100g soil.

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# SECTION 1

# INTRODUCTION

## 1.1 PURPOSE

The purpose of this document is to provide toxicity tests useful for detecting and quantifying toxicity in hazardous waste sites.

The Comprehensive Environmental Response Compensation and Liability Act (CERCLA) as amended by the Superfund Amendment and Reauthorization Act of 1986 (SARA) requires a level of control consistent with goals established by other legislation including the Safe Drinking Water Act (SDWA) and the Clean Water Act (CWA). In order to meet the requirements of the Water Acts, EPA has established acceptable limits for specific chemicals. The toxicity tests recommended in this manual assess the short-term integrated effects of complex mixtures and provide a reasonable basis for assessing the toxicity of waste products independent of existing concentration criteria.

## 1.2 ORGANIZATION OF THE DOCUMENT

This document, which is divided into two major parts, provides protocols for toxicity screening at hazardous waste sites. The first part presents issues relating to the use of toxicity tests, including background information, ongoing research, and application of tests (Section 1); data analysis and calculations and uses of toxicity indices (Section 2); sampling considerations, including the choice of media to sample and variability issues (Section 3); and finally, a hypothetical case study illustrating the use and application of the tests (Section 4).

The second part of the document is an Appendix which provides detailed, step-by-step protocols for using the toxicity tests. Recommended protocols for toxicity tests are presented for water flea survival (Daphnia magna Straus and D. pulex Leydig), algal growth (Selenastrum capricornutum Printz), fish survival (fathead minnow, Pimephales promelas Rafinesque), lettuce seed germination (Lactuca sativa L.), lettuce root elongation (Lactuca sativa), and earthworm survival (Eisenia foetida Savigny). This test battery of protocols can be applied to soils, sediments, elutriates

from soils or sediments, ground water, and surface water for the purpose of measuring short-term toxicity effects.

## 1.3 BACKGROUND

A high priority of the U.S. Environmental Protection Agency (EPA) is to identify, characterize, and clean up hazardous waste sites. These activities are regulated by CERCLA and SARA. Both CERCLA and SARA address the toxic effects of hazardous wastes, and consequently, toxicity is one of the principal characteristics used to identify and characterize hazardous waste sites.

The toxicity of wastes can be estimated using two approaches: a toxicity-based approach and/or a chemical-specific approach. In the toxicity-based approach, toxicity tests are used to measure toxicity directly. Toxicity is somewhat analogous to the biochemical oxygen demand (BOD) used in waste water analyses. Both toxicity and BOD are generic measurements of complex chemical mixtures that do not permit identification of cause-and-effect relationships. The toxicity-based approach was developed for measuring and assisting in the regulation of the toxicity of complex effluents discharged to surface waters (EPA 1985). It has also been used to identify and characterize toxic wastes under regulations enforced by the Resource Conservation and Recovery Act (RCRA) of 1976 as amended (Millemann and Parkhurst 1980).

In the chemical-specific approach, chemical analyses and water quality criteria are used to estimate toxicity. If concentrations of specific chemicals in hazardous wastes exceed criteria values, then the concentrations are considered to be toxic. The chemical-specific approach is also used for regulating waste water discharges under the Clean Water Act and for characterizing toxic wastes under RCRA.

These two approaches complement each other, and depending on site-specific conditions, either or both may be appropriate for estimating the toxicity of hazardous wastes. For complex chemical mixtures of unknown composition, such as hazardous waste site samples, however, the toxicity-based approach is generally considered to be the best procedure for estimating potential

toxicity (Bergman et al. 1986, EPA 1985). Rationale for using the toxicity-based approach rather than the chemical-specific approach includes the following:

- Toxicity tests measure the aggregate toxicity of all constituents in a complex waste mixture, including additive, synergistic, and antagonistic effects.
- The bioavailability of toxic chemicals is measured with toxicity tests but not with chemical analyses; therefore, chemical data may over- or underestimate the toxicities of single chemicals.
- Chemical analyses for complex wastes (many chemicals present), especially for organics, can be more expensive than toxicity testing.
- The specific chemicals analyzed in hazardous wastes may not include many toxic chemicals that are actually present.
- Water quality criteria are available for relatively few chemicals potentially present in hazardous wastes.
- It is not always clear from chemical data which compounds are causing toxicity in a complex hazardous waste mixture.

The chemical-specific approach may be appropriate for the following cases:

- Simple wastes (few chemicals present), for which chemical analyses can be less expensive than toxicity testing.
- Specific problem chemicals, such as carcinogens or bioaccumulative chemicals, that can be directly measured.
- Design of treatment systems, which are more easily designed to remove specific chemicals than to reduce a generic parameter such as toxicity.

This manual provides toxicity test methods for detecting and quantifying short-term toxicity in solid or liquid samples collected at hazardous waste sites. These methods are cost-effective tests that generate data useful for identifying toxic wastes, quantifying their relative toxicities, and monitoring remediation efforts. Assessing the actual or potential site-specific effects of the wastes

would require more intensive and costly toxicity tests, waste characterizations, chemical analyses, and site-specific field assessment studies.

## 1.4 ONGOING RESEARCH

Ongoing research at the Corvallis Environmental Research Laboratory (CERL) has been designed to test and evaluate the recommended toxicity test protocols on samples obtained from hazardous waste sites nationwide. The objectives of this research are to (1) improve test precision and accuracy, (2) increase the ability to detect toxicity, and (3) enhance the efficiency of prescribed laboratory procedures.

Research at CERL includes evaluating problematic situations that potentially may arise when the protocols presented here are applied under a variety of laboratory and field conditions. Specific areas of concern include (1) test solutions with low pH or high hardness that can mask the toxicity of other chemical components, (2) filtration of test solutions for toxicity tests other than the algal test, and (3) addition of ethylenediaminetetraacetic acid (EDTA) to the algal test solutions to enhance iron bioavailability.

While the recommended toxicity tests should, in general, be implemented using the protocols presented in the Appendix, the protocols should not be considered inflexible. These protocols have been developed as state-of-the-science methods for use in hazardous waste toxicity assessments. Just as the science of environmental toxicology for complex mixtures is evolving, the methods recommended in this document are expected to evolve. As new information on environmental toxicology becomes available, new techniques undoubtedly will be developed, tested, and evaluated. The methods and recommendations presented in this document will, as a consequence, be revised.

Several important factors should be considered, however, prior to modification of the recommended protocols. These factors have a strong influence on the ability of a toxicity test to measure potential toxicity. First, altering the pH or hardness of test solutions or adding EDTA to test solutions may alter the bioavailability and potential toxicity of many chemicals. For example, raising

the pH can decrease the toxicity of many metals and can increase the toxicity of ammonia and cyanide, and decrease the toxicity of hydrogen sulfide. In the same manner, increasing the hardness of test solutions adds carbonates that may complex metals, thereby reducing their toxicity. Additionally, increasing the hardness often causes increases in pH. EDTA added to test solutions can also complex metals, reducing their toxicity. Second, filtering of test materials is required only for the algal toxicity test (see Appendix, Section A.8) Filtration through a 0.45-µm filter may remove toxic particles or colloids, and may cause toxic chemicals present in trace amounts to be adsorbed onto the filter. Consequently, filtration may lead to underestimation of actual toxicity.

## 1.5 USES OF TOXICITY TESTS IN IDENTIFYING AND CHARACTERIZING THE TOXICITY OF HAZARDOUS WASTES

Toxicity data resulting from the battery of tests recommended in this document have applications in the overall assessment and remediation process of hazardous waste sites (Figure 1). Sections 1.5.1 through 1.5.5 describe these potential applications. Section 2.3 provides an example of how toxicity data can be applied to site prioritization and monitoring.

### 1.5.1 Site Prioritization

Over 24,000 uncontrolled hazardous waste sites have been identified by EPA (EPA 1986). Given this large number of sites, it is important to focus available remediation resources on those sites of greatest hazard to the environment.

Data on the toxicity of liquid or solid samples from hazardous waste sites provide a cost-effective and rapid means for ranking the potential environmental hazards of the sites. Sites are ranked based on the aggregate toxicity of the components of the complex sample, including additive, synergistic, and antagonistic effects. The indices used to rank the sites are the median lethal concentration (LC50) and the median effective concentration (EC50) (see Section 2.2). The site with the lowest LC50 or EC50 is considered to have the highest potential for toxicity, and the site with the highest LC50 or EC50 is considered to have the lowest potential for toxicity. Variability in the estimate of the toxic potential of a site, caused by both field and laboratory factors, must be

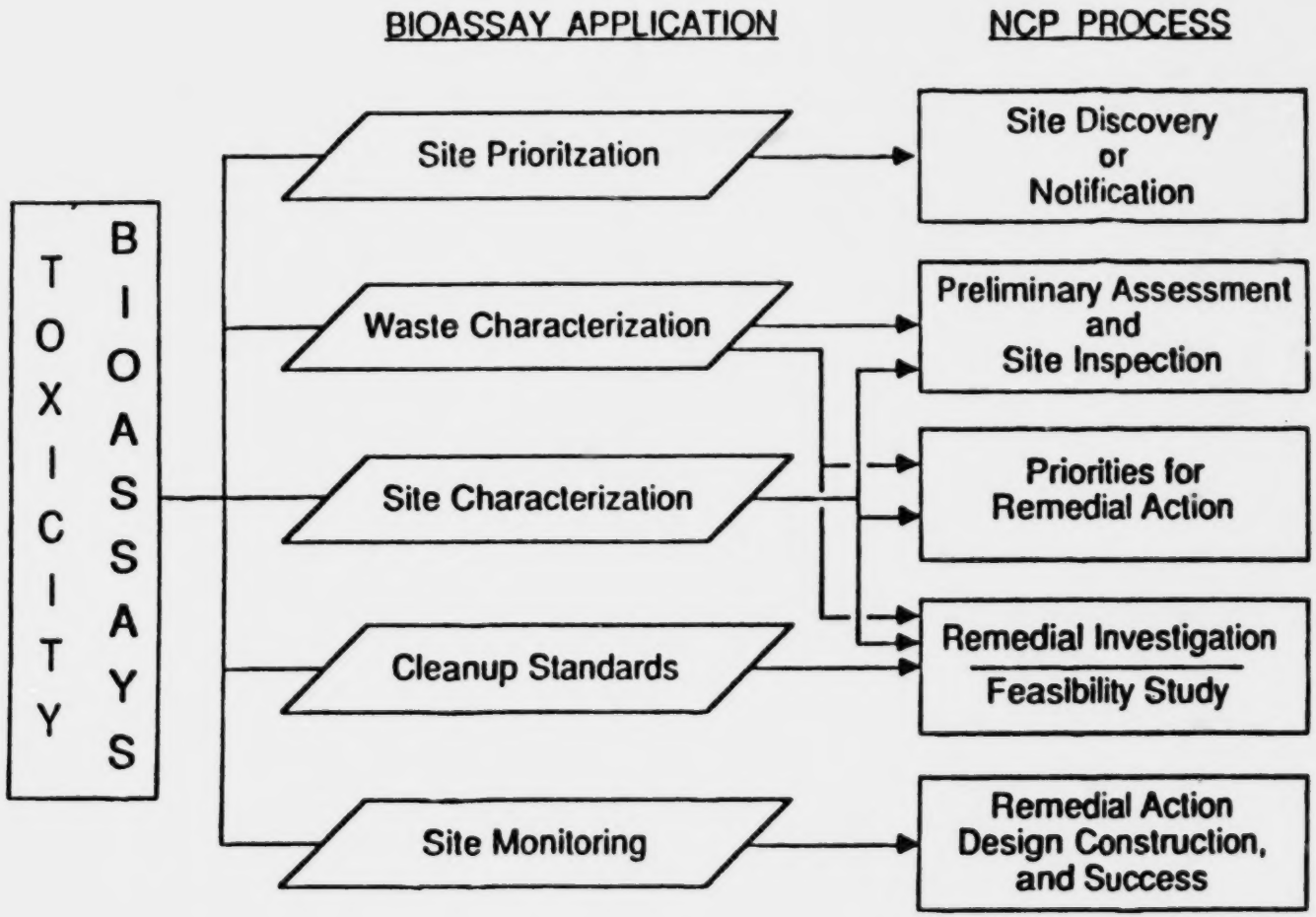


Figure 1. Role of toxicity tests in the hazardous waste site evaluation process.

considered when comparing sites (Section 2). The use of statistically-based field sampling designs and the application of a consistent set of toxicity test protocols will minimize this variability. The test battery approach recommended in this document optimizes the ability to detect toxicity at a hazardous waste site and minimizes the uncertainty in the site rankings. Differential responses among the six toxicity tests optimize the probability of detecting short-term toxicity if it is present and enable the sites to be prioritized according to the magnitude of potential short-term toxicity. Although not addressed in this document, field assessments of the potentially toxic sites would be implemented to define cause-and-effect relationships and aid in the selection of appropriate remediation measures.

### 1.5.2 Waste Characterization

Both CERCLA and RCRA require identification and characterization of wastes. These assessments are generally conducted prior to a detailed sampling program. Preliminary assessments are based on available chemical data for the site and the documented characteristics of each chemical. High costs associated with chemical analyses increase the importance of focusing detailed sampling studies on areas posing the greatest hazard. The toxicity tests recommended in this manual provide an estimate of short-term toxicity independent of chemical identification of all toxic substances.

### 1.5.3 Site Characterization

One of the most important uses of toxicity tests is for characterization of environmental conditions in and near hazardous waste sites. Site characterization involves the collection of data on hazardous substances and the assessment of potential toxicity effects at the site. Toxicity tests can form an important part of the descriptive assessments required in the site characterization process. Additionally, toxicity test results, combined with a properly designed field program, can be used to assess the extent of contamination and associated potential for toxic effects.

### 1.5.4 Cleanup Standards

The measured concentration of a toxic chemical at a hazardous waste site must be compared with all relevant criteria and standards. SARA has established that many of these standards promulgated under other Acts (e.g. SDWA, Toxic Substances Control Act [TSCA], CWA) are mandatory for site cleanup. The criteria are relevant because they are specified by law. The lack of effects-based criteria for these media and for many chemicals, and the disagreement over using criteria additively in complex chemical settings, results in major difficulties in defining cleanup levels.

Toxicity test results can provide valuable information relevant to the decision process associated with cleanup actions. The identification of potential site toxicity is an important step in the decision process. However, cleanup actions based on detailed cause-and-effect relationships require additional field assessment studies not covered in this report.

### 1.5.5 Site Monitoring

Environmental monitoring during and following remedial action is an important part of CERCLA and RCRA assessments. Toxicity tests are a valuable monitoring tool because they provide cost-effective, rapid assessment of potential toxicity. Length of exposure during toxicity testing ranges from 2 (D. magna) to 14 (earthworm) days after sample preparation. Thus, test results can be available within approximately 4 to 16 days. Monitoring can ensure that target cleanup levels are achieved and that no additional problems develop following site remediation or closure. If remediation is not completely successful at a hazardous waste site, it is important to identify the inadequacies as soon as possible to allow for implementation of additional corrective actions. As with site ranking methods (Section 1.5.1), detailed identification of cause-and-effect relationships requires additional field assessment studies. Protocols for field bioassessments are not included in this report.

## SECTION 2

## DATA ANALYSIS

This section presents methods for analyzing data obtained from implementation of recommended toxicity test protocols. Interpretation of the toxicity test results is dependent on the selected methods for analysis and presentation of the data. This section will address methods for expressing biotic response (e.g., percent mortality and inhibition), calculation of the LC50 or EC50, and recommended methods for intersite comparisons (use of the toxicity test results).

### 2.1 METHODS FOR CALCULATING MEASURES OF TOXICITY

Two measures of toxicity, specific to the type of data generated, are calculated for the recommended toxicity tests -- percent mortality and inhibition proportion. The two sections following provide methods for calculating appropriate toxicity indices.

#### 2.1.1 Percent Mortality

Percent mortality (defined for a specific toxicity test as [number of test units dying in a single sample dilution/total number of test units in the dilution] X 100) is the toxicity index calculated for four of the six toxicity tests. These tests are those using D. magna and D. pulex, fathead minnow, earthworm, and lettuce seed germination. In the lettuce seed germination test, it is recognized that factors other than the presence of a toxic substance can prevent seed germination. For the purpose of calculating the toxicity index in this test, however, lack of seed germination is considered to be equivalent to mortality.

Percent mortality is calculated for each test dilution. If the total number of test units surviving in the test dilution is equal to or greater than that of the controls, then the test solution is considered not to exhibit short-term toxicity. For any specific toxicity test, values of the calculated percent mortality at various dilutions are used to estimate the LC50 for the test (Section 2.2).

### 2.1.2 Effect Proportion

The effect proportion (E) is the toxicity index calculated for the algal and lettuce root elongation tests, and is calculated for each test dilution as:

$$(T - C) / C = E$$

where C = the mean growth in the controls, and

T = the mean growth in the test chambers at a given test dilution.

If the effect proportion is zero (C = T), then the test solution is considered not to exhibit short-term toxicity. If stimulation occurs (T > C), E will be positive, and the test solution is considered nontoxic. If inhibition occurs (T < C), E will be negative, and the test solution is considered toxic. For either the algal or lettuce root elongation test, values of the calculated inhibition proportion at various dilutions are used to calculate the EC50 for the test (Section 2.2).

If the effect proportion is zero (C = T), then the test solution is considered nontoxic. If stimulation occurs (T > C), the test solution also is considered nontoxic. For either the algal or root elongation bioassays, values of the calculated effect proportion at various dilutions are used to calculate the $EC_{50}$.

When proportional amendments of assay media are used (see Section A.8.4.11.1 of the Appendix) in the algal growth test, effect proportions are calculated for each dilution as:

$$\frac{(T - IN) - P(C - IN)}{P(C - IN)} \times 100 = E$$

where IN = dry weight (mg/L) of inoculum used at start of test, and

P = percentage volume of algal assay media used to dilute the test samples.

P should be $\geq$ 20% of the total test solution.

## 2.2 CALCULATING THE LC50 AND EC50

Data from the recommended short-term toxicity tests are used to estimate the LC50 or the EC50. The LC50, calculated for those toxicity tests measuring mortality, is the concentration that is

estimated to be lethal to 50% of the organisms within the test period. The EC50, calculated for those toxicity tests measuring a non-lethal toxic effect, is the concentration that reduces the average response of the test organisms by 50% within the test period. Only rarely will the concentration in the test dilution result in exactly 50% mortality or effect of the test organisms.

Therefore, some mathematical approach is generally needed to obtain an estimate of the LC50 or EC50. Also, because of natural variation in the sensitivity of individuals within a group of test organisms, there is a degree of uncertainty regarding the estimated value of the LC50 or EC50. This uncertainty is expressed as a confidence interval or range of values within which the "true" LC50 or EC50 could occur.

Unlike toxicity tests with single compounds, which usually result in a regular progression in percent mortality or effect with increasing toxicant concentration, toxicity tests with elutriates, soils, or complex aqueous mixtures tend to yield all-or-nothing responses. Exposures to one or more of the higher sample concentrations (lower dilutions) result in 100% mortality of the test organisms, whereas exposures at lower concentrations (higher dilutions) all result in 100% survival. These results eliminate the use of some candidate methods for calculating the LC50 or EC50 at the recommended dilutions.

Methods for calculating the LC50 or EC50 for each toxicity test include the graphical, Litchfield-Wilcoxon, binomial, Probit, Logit, moving average, moving average-angle, and Spearman-Karber analyses (Finney 1971, Stephan 1977, Peltier 1978a,b). Although any of these methods will provide reasonable estimates of the LC50 or EC50, those that also provide estimates of the confidence interval are recommended for hazardous waste site rankings. Whenever possible, methods such as the moving average-angle or Probit methods should be used because they permit the uncertainty inherent in the estimation of the LC50 or EC50 to be calculated. When estimating the LC50, EC50, or their estimators using parametric statistical methods such as simple linear regression, the investigator must be careful not to bias the results by violating any of the underlying distributional assumptions (Kennedy 1985). When the toxicity tests yield all-or-nothing results, the Litchfield-Wilcoxon, Probit, and Spearman-Karber methods cannot be used to calculate the LC50.

Step-by-step methods for calculating an LC50 can be found in Peltier and Weber (1985). Computer programs can be obtained by contacting Mr. James Dryer, U.S. EPA, Environmental Monitoring and Support Laboratory, Cincinnati, OH, 45268. For routine analysis, use of the computer programs are recommended for efficient and consistent results.

## 2.3 USE OF THE TOXICITY TEST RESULTS

Toxicity test results are appropriate for prioritizing and monitoring hazardous waste sites. The relative index of toxicity is the LC50 or EC50 and their associated confidence intervals, which are calculated from the data generated using laboratory procedures conducted according to the protocols contained in the Appendix of this document. Correct interpretation of the data is imperative for understanding and applying the toxicity test results. This section describes methods for calculating and presenting the toxicity test results.

In general, it is recommended that screening for the purpose of assessing the relative toxicity of hazardous waste sites be accomplished by implementing the entire battery of toxicity tests presented in this document. For each toxicity test performed, an LC50 or EC50 can be calculated. In most cases, the uncertainty associated with the LC50 or EC50, represented by the confidence interval, also can be calculated (Section 2.2).

The data can be presented in a manner similar to the example provided in Table 1. In this example, the toxicity test battery was applied to three hypothetical hazardous waste sites. The objective of this study was to assess the relative toxicity of each of the three sites and rank the sites with respect to their degree of toxic potential. For each test at each site, the LC50 or EC50 was calculated using the moving average-angle method. Values of LC50 or EC50 can be compared among sites only for toxicity tests that use the same organisms and protocols. Comparisons among different toxicity tests is inappropriate because the types of organisms used differ, as do the protocols, e.g., the LC50 resulting from the D. magna test, conducted for 2 days, cannot be directly compared to that for the earthworm test, conducted for 14 days. Thus, intersite comparisons must be test-specific and are made by evaluating the magnitude of the LC50s or EC50s as well as their

**TABLE 1. EXAMPLE USE OF TOXICITY TEST RESULTS. CALCULATED LC50 or EC50 AS PERCENT SAMPLE CONCENTRATION (95% CONFIDENCE INTERVAL)**

| Toxicity Test | Site 1 | Site 2 | Site 3 |
|---|---|---|---|
| D. magna[a] | 35.5 (29.4-42.5) | 32.6 (27.2-39.1) | 46.9 (42.6-51.6) |
| earthworm[a] | 6.8 (6.2-7.5) | 7.3 (6.6-8.1) | 15.6 (14.2-17.2) |
| fathead minnow[a] | 10.5 (9.5-11.6) | 10.4 (9.5-11.4) | 11.2 (10.2-12.3) |
| lettuce seed germination[a] | 45.6 (41.4-50.2) | 52.3 (47.5-57.5) | 75.7 (68.8-83.3) |
| lettuce root elongation[b] | 45.6 (38-54.7) | 54.5 (45.4-65.4) | 86.8 (78.9-95.5) |
| algal[b] | 7.3 (6.6-8.0) | 8.1 (7.3-8.9) | 14.6 (13.2-16.1) |

[a] LC50s calculated using the moving average-angle method.
[b] EC50s calculated using the moving average-angle method.

associated uncertainty, as represented by their respective confidence intervals. For this example, a 95% confidence interval was calculated. Note that although most LC50 and EC50 mean values for Site 2 were generally higher than for Site 1, the confidence intervals have a high degree of overlap. Hypothesis testing, using a two-sample "t" statistic or an appropriate multiple comparison test for example, would show no statistical differences between the LC50s and EC50s calculated for Site 1 and Site 2. No difference in the toxic potential of Site 1 or Site 2 can therefore be statistically determined. The LC50s and EC50s calculated for Site 3, however, are higher than for either Site 1 or Site 2. Additionally, with the exception of the fathead minnow test, the confidence intervals for the Site 3 indices do not overlap the corresponding confidence intervals for Sites 1 and 2. Therefore, based on the toxicity test results, Sites 1 and 2 have a higher potential for short-term toxicity than Site 3.

Conceivably, individual toxicity tests could give inconsistent results when ranking sites. For example, the earthworm toxicity test may rank Site 1 as more toxic than Site 2, with the D. magna test giving opposite results. This scenario should be expected due to the different sensitivities of the organisms in the test battery. In this case, both sites are considered to have toxic potential, possibly resulting from different chemicals or processes. In such a case, additional field assessments (not covered in this report) would be needed to assess the actual difference in toxic mechanisms between the two sites.

## SECTION 3

## SAMPLING CONSIDERATIONS

This section describes important factors for consideration when designing field sampling protocols for toxicity screening at hazardous waste sites. A properly designed sampling program is necessary to ensure that conclusions are based on reliable data, to maximize the information gained from the study while minimizing the study costs, and to ensure the repeatability and precision of the measured toxic responses. Correct interpretation of the toxicity test results is dependent upon a properly designed and implemented sampling program.

### 3.1 WHAT TO SAMPLE

The choice of which media or medium to sample in the field (soil, soil water, surface water, ground water, etc.) depends on the objectives of the study. The selected medium should maximize the capability of detecting contamination at the hazardous waste site. The media (or medium) chosen for sampling should be (1) those into which the contaminants are known or suspected to have been disposed, or (2) those to or from which the contaminants are likely to migrate. For situations in which the investigator has no prior knowledge of the contaminants or their associated spatial distribution, a general survey to obtain samples representative of the site is initially appropriate. Resources permitting, additional surveys, the design of which can be guided using the results of toxicity testing on the first set of samples, can aid in further investigating the potential toxicity of the hazardous waste site. Field assessments of the hazardous waste site are necessary for evaluating cause-and-effect relationships. Appropriate techniques for bioassessment are not presented in this report.

### 3.2 VARIABILITY ISSUES

A major objective of the general surveys is to minimize variability associated with data collection while maximizing the ability to detect site toxicity. Variability of data derived from field

samples, collected during the survey of a hazardous waste site, is attributable to a number of different factors. Sampling variability can be attributed to natural variability, variability in field conditions when the samples are taken, sampling techniques, and sample handling in the laboratory. Although the natural variability among samples taken at the same time and place cannot be controlled by the investigator, some part of it may be anticipated and accounted for by a statistically-based field sampling design.

Concentrations of contaminants usually vary both spatially and temporally in the field. Spatial variability tends to be higher in soils and sediments than in water, because soil/sediment mixing rates are much slower than those in water. Conversely, temporal variability in water may be greater than in soils and sediments because transport of material is more rapid in water than in soils/sediments.

To account for spatial and temporal variability in concentrations of contaminants, statistically-based sampling designs should be implemented for the collection of samples at hazardous waste sites. Several types of statistical designs and the advantages and disadvantages of each for assessing potential toxicity at hazardous waste sites can be found in Athey et al. (1987).

## SECTION 4

## CASE STUDY

The case study presented in this section is an example selected to illustrate the use of the toxicity tests presented in this manual. The example provided illustrates the procedures and techniques recommended to identify and quantify short-term toxicity of hazardous waste site samples.

### 4.1 BACKGROUND INFORMATION

The hazardous waste site is an abandoned railroad tie treatment plant. The site was known to be contaminated with creosote and other wood-preserving materials. A preliminary site visit was made to obtain background information on the history of chemical disposal, determine the dimensions of the site, and define any special sampling problems. The site is bounded on the east and north by a river.

Records obtained from the local department of natural resources indicate that creosote and, occasionally, pentachlorophenol were used on the site for wood treatment. Minimal cleanup had occurred before the site was closed. It was clear from the visit that creosote was still being emitted from the bank into the water along some parts of the river. Piles of creosote-contaminated material, as well as pools of black sludge, were located near ponds immediately adjacent to the storage tanks on the south side of the site.

### 4.2 STUDY OBJECTIVES

The objective of the study was to identify and quantify the short-term toxicity, if any, of contaminated water and sediments at the site. If the toxicity tests identified significant toxicity in site samples, a more intensive field study would be designed to supply data needed for assessment of ecological damage (methods are not addressed in this report).

## 4.3 STUDY PROCEDURES

A bridge was chosen as the starting point for sample location. Water and sediment samples were collected at 660 m west of the bridge (farthest downstream location), 380 m west of the bridge, 200 m west of the bridge, 120 m east of the bridge, 220 m east of the bridge (farthest upstream location), and 3000 m upstream (the control position). In addition, sediment samples were taken from ponds near the storage tanks. The sampling points were chosen to bracket the visibly contaminated zone. The sampling design was reviewed before the field work began by the project manager, a statistician, the field sampling crew, and the toxicologist in charge of laboratory analyses.

Protective clothing and masks were worn by the field sampling personnel during sample collection.

## 4.4 ANALYSIS AND EVALUATION

The results of the toxicity tests are presented in Table 2. The only sites with measurable short-term toxicity are the ponds and those sites 660 and 380 m west of the bridge. The water sample from the 380-m west site is highly toxic, while the sediments from that location exhibit lower toxicity. At the 660-m west site, however, the sediments are highly toxic to some organisms while the water exhibits no measurable short-term toxicity. The results of the toxicity testing indicate at least the potential for ecological effects from contaminated site water and sediments. From the perspective of site prioritization, upstream sites exhibit negligible short-term toxicity, while sites at 380 m and 660 m downstream exhibit significant short-term toxicity.

Following remedial action at the site, which included sediment removal, the toxicity tests were again used to determine if the methods for removing the short-term toxic contamination were effective. The battery of toxicity tests was applied to sediment and water samples taken at the same locations as the original samples. The toxicity test results indicated no short-term toxicity. Periodic toxicity testing was then implemented to monitor for the possibility of future toxic contamination.

The toxicity tests cannot identify the actual causes of ecological effects at the site. A comparison of the results of Table 2 suggests that water soluble toxic compounds in sediments from this site may be causing significant ecological effects at substantial distances downstream. It was recommended that ecological assessment studies should be undertaken at these downstream sites.

## TABLE 2. TOXICITY TEST RESULTS FROM SAMPLES COLLECTED AT THE RAILROAD TIE TREATMENT PLANT

| Sample Location | Sample Type | LC50 or EC50[f] (95% Confidence Interval) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Algae[a] | D. magna | Fathead Minnow[a] | Lettuce Root Elongation[a] | Lettuce Seed Germination[b] | Earthworm[b] |
| 660 m west | Sediment | 63.7 (57.9 - 70.1) | 73.6 (66.9 - 90) | 4.0 (3.6 - 4.4) | 100 (NA)[e] | 70.3 (63.9 - 77.3) | 27.9 (25.4 - 30.7) |
| | Water | NE[c] | NE | NE | NE | ND[d] | ND |
| 380 m west | Sediment | 73.7 (67 - 81.1) | NE | 29.9 (27.2 - 32.9) | 100 (NA) | 100 (NA) | 27.9 (23 - 33.5) |
| | Water | 6.6 (6 - 7.3) | 0.2 (.18 - .22) | 9.6 (8.7 - 10.6) | 100 (NA) | ND | ND |
| 200 m west | Sediment | NE | NE | NE | NE | NE | NE |
| | Water | NE | NE | NE | NE | NE | NE |
| 120 m west | Sediment | NE | NE | NE | NE | NE | NE |
| | Water | NE | NE | NE | NE | NE | NE |
| 220 m west | Sediment | NE | NE | NE | NE | NE | NE |
| | Water | NE | NE | NE | NE | ND | ND |
| 3000 m upstream | Sediment | NE | NE | NE | NE | NE | NE |
| | Water | NE | NE | NE | NE | ND | ND |
| Ponds | Sediment | 0.6 (.5 - .67) | 6.9 (6.3 - 7.6) | 8.5 (7.7 - 9.4) | 8.1 (7.4 - 8.9) | 0.9 (.82 - .99) | 3.9 (3.5 - 4.3) |

[a] Tests conducted with sediment elutriates.
[b] Tests conducted with sediment samples.
[c] NE = No effect.
[d] ND = Test not done.
[e] NA = Not applicable, 95% confidence interval could not be calculated.
[f] Percent test solution.

## SECTION 5

## REFERENCES


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## APPENDIX A -- TOXICITY TEST METHODS

### SECTION A.1

### SCOPE AND APPLICATION

Procedures are described for obtaining information on the acute toxicity of hazardous wastes to aquatic and terrestrial organisms. Six static acute toxicity tests are described: algal growth (Selenastrum capricornutum), water flea survival (Daphnia pulex and D. magna), fish survival (fathead minnow, Pimephales promelas), earthworm survival (Eisenia foetida), seed germination (lettuce, Lactuca sativa), and lettuce root elongation. These procedures are derived from methods in common use for testing the acute toxicity of effluents and single chemicals (e.g., EPA 1982, ASTM 1980, Peltier and Weber 1985, Horning and Weber 1985, Edwards 1984, Thomas and Cline 1985). Only minor modifications of these methods have been made to adapt them for tests with hazardous wastes.

The tests can be performed with both aqueous and solid hazardous waste samples. The algae, water flea, fish, and lettuce root elongation tests are for use with aqueous waste samples or elutriates prepared from solid wastes. The earthworm and lettuce seed germination tests are for testing hazardous wastes mixed with artificial soil. If desired, the earthworm and lettuce seed germination tests could be performed with artificial soils hydrated with aqueous samples or solid waste elutriates.

These toxicity test methods can be used to test the toxicity of other kinds of solid and aqueous materials collected at hazardous waste sites, such as surface waters, ground waters, soils, and sediments.

Procedures are described for obtaining and culturing the test organisms, for handling and storing hazardous wastes, and for preparing the elutriates and artificial soils required for toxicity testing.

These toxicity test methods are state-of-the-science procedures. Because the state-of-the-science for environmental toxicology is continually evolving, methods for performing these tests also will evolve and improve. Therefore, these methods may be revised as better methods are developed.

Some of the methods have undergone more development, testing, and standardization than others. For example, the aquatic tests described in this report have been in widespread use in governmental agencies and private companies for quite some time, while the earthworm test was only recently introduced, has not been standardized, and is much less routinely used. Consequently, the earthworm test may undergo more changes and refinement than other tests.

## SECTION A.2

## HEALTH AND SAFETY

### A.2.1 GENERAL PRECAUTIONS

Collection and use of hazardous wastes for toxicity testing may involve significant risks to personal safety and health. Personnel collecting hazardous waste samples and conducting toxicity tests should take all safety precautions necessary for the prevention of (1) bodily injury and illness which might result from ingestion or invasion of infectious agents, (2) inhalation or absorption of corrosive or toxic substances through skin contact, and (3) asphyxiation due to lack of oxygen or presence of noxious gases.

Before sample collection and laboratory work begin, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition. In addition, all laboratories conducting toxicity testing on hazardous wastes should prepare a "Safety and Health Protocol for Shipping, Receiving, Handling and Testing of Toxic Substances." This protocol document should be consulted for laboratory-specific instructions on health and safety procedures. To obtain an example of such a protocol, contact James C. McCarty, CERL Designated Safety and Health Official, Environmental Research Laboratory, U.S. Environmental Protection Agency, 200 SW 35th Street, Corvallis, OR 97333.

### A.2.2 SAFETY MANUALS

For further guidance on safe practices when collecting hazardous waste samples and conducting toxicity tests, consult general industrial safety manuals, including Walters and Jameson (1984) and NIOSH et al. (1985).

## SECTION A.3

## QUALITY ASSURANCE

### A.3.1 INTRODUCTION

Quality assurance (QA) practices for hazardous waste toxicity tests consist of all aspects of the test that affect data quality: (1) hazardous waste sampling and handling, (2) the source and condition of the test organisms, (3) condition of equipment, (4) test conditions, (5) instrument calibration, (6) replication, (7) use of reference toxicants, (8) record keeping, and (9) data evaluation. The QA guidelines presented here are adapted from Horning and Weber (1985). For general guidance on good laboratory practices related to toxicity testing, see FDA (1978), EPA (1979c, 1980a, and 1980b), and DeWoskin (1984).

### A.3.2 HAZARDOUS WASTE SAMPLING AND HANDLING

Hazardous waste samples collected for on-site and off-site testing must be preserved as described in Section A.7, Hazardous Waste Sampling and Handling.

### A.3.3 TEST ORGANISMS

The test organisms used in the procedures described in this manual are the green alga, S. capricornutum; water fleas, D. magna and D. pulex; the fathead minnow, P. promelas; the earthworm, E. foetida; and lettuce, L. sativa.

### A.3.4 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

Laboratory temperature control equipment must be adequate to maintain recommended temperatures for test water. Recommended materials must be used for fabrication of the test equipment that comes in contact with hazardous waste materials (see Section A.4, Facilities and Equipment).

### A.3.5 ANALYTICAL METHODS

Routine chemical and physical analyses must include established QA practices outlined in Agency methods manuals (EPA 1979a,b).

### A.3.6 CALIBRATION AND STANDARDIZATION

Instruments used for routine measurements of chemical and physical parameters, such as pH, dissolved oxygen (DO), temperature, conductivity, alkalinity, and hardness, must be calibrated and standardized according to instrument manufacturers' procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1, EPA 1979b).

Wet chemical methods used to measure hardness and alkalinity must be standardized according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1, EPA 1979b).

### A.3.7 DILUTION WATER

The dilution water used in the toxicity tests should be synthetic soft water, except for tests with D. magna, which use moderately hard water (see Sections A.6, Dilution Water, and A.8, Toxicity Test Methods).

### A.3.8 TEST CONDITIONS

Water and air temperatures must be maintained within the limits specified for each test. Dissolved oxygen concentrations for aquatic tests and pH of soils and solutions should be checked at the beginning of the test and end of the test period.

### A.3.9 TEST ACCEPTABILITY

The results of the reference toxicant tests are unacceptable if mean control survival is less than 90%. The results of the definitive toxicity tests are also unacceptable if control survival is less than 90%. The results of the algal toxicity test are unacceptable if the cell density in the controls after 96 h is less than $10^6$ cells/mL.

An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the test (see test condition summaries in each test method section). The acceptability of the test will depend on the best professional judgment and experience of the investigator. The deviation from test specifications must be noted when reporting data from the test.

### A.3.10 PRECISION

The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure hazardous waste toxicity. The single laboratory precision of each type of test to be used in a laboratory should be determined by performing five or more tests with a reference toxicant. Precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated end points from the replicated tests. Factors that can affect test precision include test organism age, condition, and sensitivity; temperature control; and feeding.

### A.3.11 REPLICATION AND TEST SENSITIVITY

The sensitivity of the tests depends in part on the number of replicates, the probability level selected, and the type of statistical analysis. The minimum recommended number of replicates is discussed in each test method section. The sensitivity of the test will increase as the number of replicates increases.

### A.3.12 QUALITY OF TEST ORGANISMS

If the laboratory does not have an ongoing culturing program for test organisms and obtains them from an outside source, the sensitivity of each batch of test organisms must be evaluated. Evaluations are performed with a reference toxicant in a toxicity test run concurrently with the hazardous waste toxicity tests. If the laboratory maintains breeding cultures, the sensitivity of the offspring must be determined in a toxicity test performed with a reference toxicant at least once each month. If preferred, this reference toxicant test may be performed concurrently with the hazardous waste toxicity test.

A 24-h acute toxicity test is used to determine the sensitivity of fathead minnows and D. spp. For all other test species, tests of the standard length described in each method are used to determine the sensitivity of the test organisms.

Three reference toxicants are available from EMSL-Cincinnati to establish the precision and validity of toxicity data generated by testing laboratories: sodium dodecylsulfate (SDS), sodium pentachlorophenate (NaPCP), and cadmium chloride ($CdCl_2$). The reference toxicants may be obtained by contacting the Quality Assurance Branch, Environmental Monitoring and Support

Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268; FTS 684-7325, commercial 513-569-7325. Instructions for the use and the expected toxicity values for the reference toxicants are provided with the samples.

To ensure comparability of QA data on a national scale, all laboratories must use the same source of reference toxicant (EMSL-Cincinnati) and the same formulation of dilution water -- soft synthetic water (moderately hard synthetic water for tests with D. magna), described in Section A.6 (Dilution Water), and algal growth medium, described in Section A.8.4 for S. capricornutum. For the lettuce seed germination and earthworm tests, reference toxicity tests should be performed using 100% artificial soil and test concentrations of the reference toxicant diluted in the deionized water used to hydrate the soil.

### A.3.13 QUALITY OF FOOD FOR TEST ORGANISMS

The quality of the food for fish and invertebrates is an important factor in toxicity tests. Suitable trout chow, brine shrimp (Artemia), and other foods must be obtained as described in Sections A.5.2-A.5.6. Limited quantities of reference Artemia cysts, information on commercial sources of good quality Artemia cysts, and procedures for determining cyst suitability as food are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268. The suitability of each new supply of food must be determined in a side-by-side test in which the response of test organisms fed with the new food is compared with the response of organisms fed a reference food or a previously used, satisfactory food.

### A.3.14 CONTROL CHARTS

A control chart should be prepared for each reference-toxicant-organism combination, and successive toxicity values should be plotted and examined to determine if the results are within prescribed limits (Figure A-1). In this technique, a running plot is maintained for the toxicity values ($X_i$) from successive tests with a given reference toxicant.

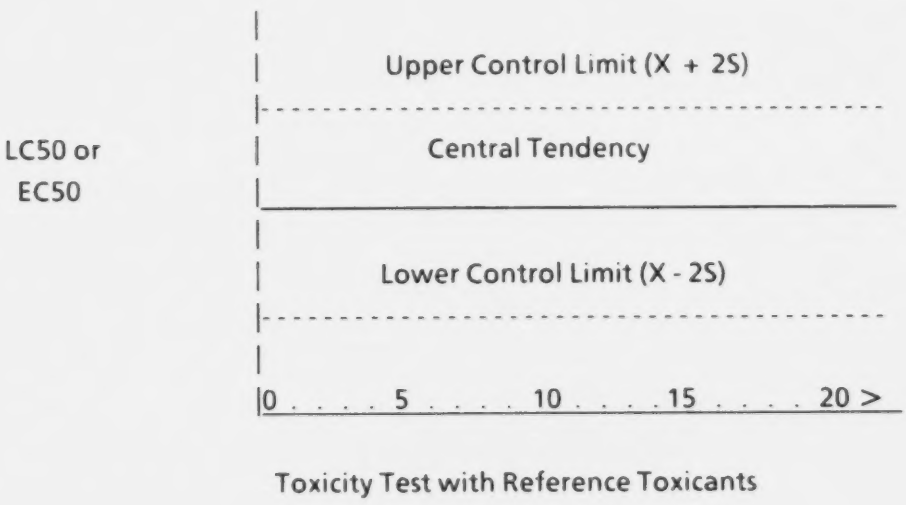
Upper Control Limit (X + 2S)
LC50 or
EC50
Central Tendency
Lower Control Limit (X - 2S)
0 . . . . 5 . . . . 10 . . . . 15 . . . . 20 >
Toxicity Test with Reference Toxicants

**Figure A-1. Control chart.**

The type of control chart illustrated (EPA 1979a) is used to evaluate the cumulative trend of the statistics from a series of samples. The mean (X) and upper and lower control limits ( $\pm$ 2S) are recalculated with each successive point, until the statistics stabilize. Outliers, those values that fall outside the upper and lower control limits, and trends of increasing or decreasing sensitivity are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone.

If the toxicity value from a given test with the reference toxicant does not fall in the expected range for the test organisms when using the standard dilution water, then the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

### A.3.15 RECORD KEEPING

Proper record keeping is required. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt, and other pertinent information relating to their history and health, and information on the calibration of equipment and instruments, test conditions employed, and test results. Annotations should be kept current to prevent the loss of information.

## SECTION A.4

## FACILITIES AND EQUIPMENT

This section was adapted from Horning and Weber (1985).

### A.4.1 GENERAL REQUIREMENTS

#### A.4.1.1 Laboratory Facilities

Hazardous waste toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing, holding, and acclimating organisms. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, dechlorinated tap water (see Section A.6, Dilution Water), or synthetic water. Dechlorination can be accomplished by aeration (allowing the water to stand in an open vessel for 24 h), carbon filtration, or the use of sodium thiosulfate. Use of 1.0 mg (anhydrous) sodium thiosulfate/L will reduce the chlorine content of 1.5 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and fumes. Test facilities must be well ventilated and free of fumes. During rearing, holding, acclimating, and testing, test organisms should be shielded from external disturbances.

#### A.4.1.2 Materials

Materials used for exposure chambers, tubing, etc., that come in contact with hazardous waste materials, should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON$^{R}$) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Plastics such as polyethylene, polypropylene, polyvinyl chloride, TYGONR, etc., may be used for test chambers or to store test materials, but caution should be exercised in their use because they could introduce toxicants when new, or carry over toxicants from one test to another, if reused. The use of glass carboys is discouraged for safety reasons.

New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) that cannot be discarded after each use, because of cost, must be decontaminated according to the cleaning procedures listed in Section A.4.3, Cleaning. Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with the test media before use in the test. Copper, galvanized material, rubber, brass, and lead must not come in contact with holding, acclimation, or dilution water, or with hazardous waste and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

#### A.4.1.3 Adhesives

Silicone adhesive used to construct glass test chambers adsorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

### A.4.2 TEST CHAMBERS

Test chamber size and shape vary according to the size of the test organism. Requirements are specified in each test.

### A.4.3 CLEANING

New plasticware used for sample collection or organism exposure vessels does not require rigorous cleaning. It is sufficient to rinse the new containers once with sample before use. New glassware, however, should be soaked overnight in acid (see below).

It is recommended that all sample containers, test vessels, pumps, tanks, and other equipment that has come in contact with test materials be washed after use in the manner described below to remove surface contaminants. Special cleaning requirements for glassware used in algal toxicity tests are described in Section A.8.4.

1. Soak 15 min, and scrub with detergent in tap water, or clean in an automatic dishwasher.

2. Wash with nonphosphate detergent.

3. Rinse once with full-strength acetone to remove organic compounds.

4. Rinse twice with tap water.

5. Carefully rinse once with fresh dilute (20% V:V) nitric acid or hydrochloric acid to remove scale, metals, and bases. To prepare a 20% solution of acid, add 20 mL of concentrated acid to 80 mL of distilled water. Neutralize with sodium carbonate.

6. Rinse well with tap water.

7. Rinse twice with deionized water.

8. Baked at 400°C for 10 hours to remove residual organic contaminants.

All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

## SECTION A.5

## TEST ORGANISMS AND CULTURE METHODS

### A.5.1 INTRODUCTION

The organisms used in the toxicity tests described in this manual are the fathead minnow, P. promelas, the water fleas D. pulex and D. magna, the green alga, S. capricornutum, the earthworm, E. foetida, and lettuce, L. sativa. These organisms are easily cultured and maintained in the laboratory. Culturing, care, and handling procedures for D. spp., fathead minnows, earthworms, brine shrimp (which are required for culturing fathead minnows), and S. capricornutum are described in Sections A.5.2- A.5.5, respectively.

Starter cultures of S. capricornutum are available from the following sources:

1. Aquatic Biology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

2. American Type Culture Collection (Culture No. ATCC 22662), 12301 Parklawn Drive, Rockville, MA 10852.

3. Culture Collection of Algae, Botany Department, University of Texas, Austin, TX 78712.

Starter cultures of the fathead minnow (P. promelas) and D. spp. can be obtained from the Aquatic Biology Section, Biological Methods Branch, EMSL-Cincinnati Newtown Facility, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Newtown, OH 45244 (Phone: FTS 778-8350; Commercial 513-527-8350). Many states have strict regulations regarding the importation of non-native fishes. Required clearances should be obtained from state fisheries agencies before arrangements are made for the interstate shipment of fathead minnows.

Starter cultures of the earthworm, E. foetida, are available from Mr. Julian Stewart, Vittor & Associates, 8100 Cottage Hill Road, Mobile, AL 36695.

Lettuce seeds (L. sativa; butter crunch variety) are available from commercial seed suppliers.

If there is any uncertainty concerning the identity of the organisms, it is advisable to have them identified by a second party. Test organisms must be destroyed after use.

### A.5.2 CULTURE METHODS FOR D. SPP. (D. MAGNA AND D. PULEX)

The culture methods for D. spp. are taken from Peltier and Weber (1985).

#### A.5.2.1 Geographical and Seasonal Distribution

D. magna is principally a lake dweller and is restricted to waters in northern and western North America with hardness values >150 mg/L (as $CaCO_3$) (Pennak 1978). D. pulex is principally a pond dweller, but also is found in lakes. It is found over most of the North American continent in both hard and soft waters.

### A.5.2.2 Life Cycle

The life span of D. spp., from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (Pennak 1978). The average life span of D. magna is about 40 days at 25°C, and about 56 days at 20°C. The average life span of D. pulex at 20°C is approximately 50 days.

In culture, a female produces a clutch of 10 to 30 eggs. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton or carapace). D. pulex has three to four juvenile instars, whereas D. magna has three to five instars.

D. magna usually has six to 22 adult instars, and D. pulex has 18 to 25. In general, the duration of instars increases with age, but also depends on environmental conditions. Culture conditions should assure that ephippia are not induced. A given instar generally lasts approximately two days under favorable conditions, but when conditions are unfavorable, it may last as long as a week. The number of young per brood is highly variable for D. spp., depending primarily on food availability and environmental conditions. D. magna and D. pulex may both produce as many as 30 young during each adult instar. The number of young released during the adult instars of D. pulex reaches a maximum at the tenth instar, after which there is a gradual decrease (Anderson and Zupancic 1937). The maximum number of young produced by D. magna occurs at the fifth adult instar, after which it decreases (Anderson and Jenkins 1942).

### A.5.2.3 Morphology and Taxonomy

D. pulex attains a maximum length of approximately 3.5 mm, whereas D. magna is much larger, attaining a length of 5.0 to 6.0 mm. However, these two species can be differentiated with certainty only by determining the size and number of spines on the postabdomenal claws, using a dissecting or compound microscope (see Pennak 1978).

### A.5.2.4 Culturing Methods

#### A.5.2.4.1 Sources of Organisms--

D. spp. are available from the Environmental Monitoring and Support Laboratory-Cincinnati, and from commercial biological supply houses. Only a small number of organisms (20-30) are needed to start a culture. D. pulex is preferred over D. magna by some biologists because it is more widely distributed than D. magna and is easier to culture. However, the neonates of D. magna are larger and, therefore, somewhat easier to use in toxicity tests.

#### A.5.2.4.2 Culture Media--

Although D. spp. stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, if possible the use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it produces predictable results, and (4) it allows adequate growth and reproduction. Reconstituted moderately hard water (hardness: 80 to 100 mg/L $CaCO_3$) is recommended for D. magna, whereas soft reconstituted water (hardness: 40 to 48 mg/L $CaCO_3$) is recommended for D. pulex. If D. spp. cultures cannot be maintained in reconstituted water, prepare the culture medium from well water, surface water, or dechlorinated tap water. Adjust the water to the desired hardness by (1) diluting with deionized or distilled water to decrease hardness, or (2) adding reconstituted hardwater to increase hardness. The preparation of the media (Table A-1) follows.

**TABLE A-1. MEDIA FOR D. MAGNA AND D. PULEX PREPARED FROM SYNTHETIC WATER**

| Reagent | Concentration (mg/L) D. magna[a] | D. pulex[b] |
|---|---|---|
| $NaHCO_3$ | 96 | 48 |
| $CaSO_4 \cdot 2H_2O$ | 60 | 30 |
| $MgSO_4$ | 60 | 30 |
| KCl | 4 | 2 |

[a] Moderately hard reconstituted water medium.
[b] Soft reconstituted water medium.

The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is approximately 8.0, but it will rise as much as 0.5 unit as the D. spp. population increases. Although D. spp. can survive over a wide pH range, the optimum range is 7.0 to 8.6 (Lewis and Weber 1985). The pH of culture media should be monitored at least twice weekly.

If synthetic water does not prove to work, culture water can be prepared using dechlorinated tap water, well water, or a surface water which has been adjusted for hardness. The minimum criterion for acceptable culture water is one in which healthy organisms survive for the duration of testing without showing signs of stress.

When required, the reagents listed below can be used to increase hardness as follows:

$$\text{DELTA TOTAL HARDNESS (DTH, mg/L)} = \text{DESIRED HARDNESS} - \text{SAMPLE HARDNESS}$$

$$\text{MULTIPLICATIVE FACTOR (MF, g)} = \frac{[(\text{DTH, mg/L})(\text{DESIRED VOLUME IN LITERS})]}{1000 \text{ mg/g}}$$

Multiply each of the following constants by the multiplicative factor, and add to the desired total dilution or culture water volume in liters. Stir for at least 1 hour, or until dissolved.

| | 50 mg/L Hardness | 100 mg/L Hardness | 200 mg/L Hardness |
|---|---|---|---|
| $MgCl_2 \cdot 6H_2O$ | 0.593 | 0.593 | 0.593 |
| $CaSO_4 \cdot 2H_2O$ | 1.219 | 1.219 | 1.219 |
| $NaHCO_3$ | 1.180 | 1.230 | 1.280 |
| $KHCO_3$ | 0.120 | 0.147 | 0.236 |

EXAMPLE: The deionized water hardness is 0 mg/L $CaCO_3$; the desired hardness for the culture water is 100 mg/L $CaCO_3$; the total volume of culture water required is 2 L.

$$\mathbf{DTH} = 100 - 0 = 100 \text{ mg/L}$$

$$\mathbf{MF} = \frac{[(100 \text{ mg/L})(2 \text{ L})]}{1000 \text{ mg/g}} = 0.20\text{g}$$

The salts added to 2 L of solution to obtain a total hardness of 100 mg/L $CaCO_3$ are:

$MgCl_2.6H_2O$ (0.593 X 0.20 g **MF**) = 0.1186 g
$CaSO_4.2H_2O$ (1.219 X 0.20 g **MF**) = 0.2438 g
$NaHCO_3$ (1.230 X 0.20 g **MF**) = 0.2460 g
$KHCO_3$ (0.147 X 0.20 g **MF**) = 0.0294 g

**A.5.2.4.3 Feeding: Quantity and Frequency--**

Food preparation and feeding are of great importance in D. spp. culturing. D. spp. can be cultured using algae or a prepared food consisting of a suspension of a trout chow, alfalfa, and yeast. The latter diet is easily prepared and provides adequate nutrition for organisms used in acute toxicity tests (Winner et al. 1977). The trout chow must conform to Fish & Wildlife Service Specification PR(11)-78, and can be obtained through livestock feed stores. Dried yeast, such as Fleischmann's, can be obtained at any grocery store. Dried alfalfa can be obtained at health food stores.

The trout chow, alfalfa, and yeast food is prepared as follows:

1. Place 6.3 g of trout chow pellets, 2.6 g of dried yeast, and 0.5 g of dried alfalfa in a blender.
2. Add 500 mL of distilled or deionized water.
3. Mix at high speed for 5 min.
4. Place in a refrigerator and allow to settle for 1 h.
5. Decant the top 300 mL and save; discard the remainder.
6. Place 30- to 50-mL aliquots in small (50- to 100-mL) polyethylene bottles with screw caps and freeze.
7. Thaw portions as needed. After thawing, keep in refrigerator for a maximum of one week, then discard.

Feed 1.5 mL prepared food per 1000 mL of medium, three times per week, i.e., Monday, Wednesday, Friday. There may be some excess food in the medium at this rate of feeding, but if the medium is aerated continuously and replaced each week, as discussed below, this should cause no problems.

An alternate method of feeding D. spp. used successfully at CERL involves the use of S. capricornutum, the green alga employed in the algal growth test (described in Section A.8.4).

Daphnia are fed Monday through Thursday from a stock culture of S. capricornutum in a quantity sufficient to provide a final concentration of 2 mg/L dry weight (approximately 135,000 cells/ml) in the rearing jars which contain 2 L water and 30 to 40 adult D. magna. On Fridays the quantity is doubled to allow sufficient nutrients to sustain the cultures through the weekend without additional feeding is continuously.

Selenastrum capricornutum, cultured as a food source for Daphnia. The algae are incubated in an environmental chamber held at 24 ± 2°C with a continuous light intensity of 4304 ± 430 lux. An algal nutrient solution is prepared by adding 1.0 ml of each of the macronutrient and micronutrient stock solutions in the order listed in Table A-2, to 900 ml of filter-sterilized deionized water, with mixing after each addition. The final volume is brought to 1 liter with filter-sterilized deionized water. Deionized water (not the culture medium) is filter-sterilized by passing through a 0.22 µm porosity cellulose membrane filter (pre-rinsed with 100 ml deionized water) into a sterile container.

After the algal growth medium is prepared it is autoclaved 15 minutes/L at 121°C. A final spike of 1 ml/L BME vitamin solution (Sigma Chemical Co.) is added upon introduction of the medium into the aspirator bottle.

D. magna are fed, in addition to the algal cells suspensions, a fish-food and yeast solution at the rate of 0.2 mls per 2-L jar. This nutrient solution is fed (on Tuesdays and Fridays) after the water in the culture jars has been changed. The fish-food yeast formula is prepared by adding 12 g fish-food (e.g., salmon or trout pellets) and 3 grams bakers yeast to 1 L deionized or distilled water.

**A.5.2.4.4 Culture Temperature--**

D. spp. can be cultured successfully over a wide range of temperatures, but should be protected from sudden changes in temperature, which may cause death. The optimum temperature is approximately 22°C, and if ambient laboratory temperatures remain in the range of 18 to 26°C, normal growth and reproduction of D. spp. can be maintained without special temperature control equipment.

**A.5.2.4.5 Illumination--**

The variations in ambient light intensities (540 to 1080 lux) and prevailing day/night cycles in most laboratories do not seem to affect D. spp. growth and reproduction significantly. However, a minimum of 16 h of illumination should be provided each day.

**A.5.2.4.6 Culture Vessels--**

Culture vessels of clear glass are recommended since they allow easy observation of the D. spp. Maintain several (at least five) culture vessels, rather than only one. This will ensure backup cultures so that, in the event of a population "crash" in one or several chambers, the entire D. spp. population will not be lost. If a 3-L vessel is stocked with 30 D. spp., it will provide approximately 300 young each week.

Initially, all culture vessels should be washed well (see Section A.4, Facilities and Equipment). After the culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to rid the vessel of accumulated food and dead D. spp. (see Section A.5.2.4.8). Once per month wash each vessel with detergent during medium replacement. Rinse three times with tap water and then with culture medium to remove all traces of detergent.

**A.5.2.4.7 Aeration--**

D. spp. can survive when the dissolved oxygen (DO) concentration is as low as 3 mg/L, but do best when the level is above 6 mg/L. Each culture vessel should be continuously and gently aerated if DO levels fall below 6 mg/L. This is best accomplished by using air stones. The air can come from either an aquarium air pump or from a general laboratory compressed air supply. If the laboratory air supply is used, first pass it through a flask full of cotton batting to filter out oil or other contaminants.

**A.5.2.4.8 Weekly Culture Media Replacement--**

Careful culture maintenance is essential. The medium in each stock culture vessel should be replaced each week with fresh medium. This can be best accomplished as follows:

1. Siphon the old medium out, using plastic tubing (6 mm) covered with fine mesh netting around the open end.

2. Retain 1/10 (300 mL) of the original medium containing the D. spp. population.

3. Pour this old medium containing the D. spp. into a temporary holding vessel.

4. Clean the culture vessel as described above.

5. Fill the newly cleaned vessel with fresh medium. Gently transfer by pouring the contents of the temporary holding vessel (old medium and D. spp.) into the vessel containing the fresh medium.

If the medium is not replaced weekly, waste products will accumulate which could cause a population crash or the production of males and/or sexual eggs.

D. spp. populations should be thinned weekly to about 8 per liter to prevent over-crowding, which may cause a population crash or the production of males and/or ephippia. A good time to thin the populations is during medium replacement. To transfer D. spp., use a 15-cm disposable, jumbo bulb pipet, or 10-mL "serum" pipet that has had the delivery end cut off and fire polished. The diameter of the opening should be approximately 5 mm. When using a serum pipet, a pipet bulb (such as a Propipet$^{R}$) or a portable, motorized pipettor (Mopet$^{R}$) provides the controlled suction needed when selectively collecting D. spp. Alternatively, Daphnia may be transferred by screening.

Liquid containing adult D. pulex and D. magna can be poured from one container to another without risk of air becoming trapped under their carapaces. However, the very young D. spp. are much more susceptible to air entrapment and, for this reason, should be transferred from one container to another using a pipet. The tip of the pipet should be kept under the surface of the liquid when the D. spp. are released.

Each culture vessel should be covered to exclude dust and dirt and minimize evaporation.

**A.5.2.5 Test Organisms**

D. magna or D. pulex, 2- to 24-h in age (neonates, or first instars), are to be used in the tests. To obtain the necessary number of young for a test, remove adult females bearing embryos in their brood pouches from the stock cultures 24 h preceding the initiation of the test. Place them in 400-mL beakers containing 300 mL of medium and 0.5 mL of prepared food (see Section A.5.2.4.3). The young that are found in the beakers the following day are used for the toxicity test. Five beakers, each containing 10 adults, usually will supply enough first instars for one toxicity test.

Since the appearance of ephippia in cultures generally is indicative of unfavorable conditions, D. spp. used for toxicity tests should not be taken from cultures that are producing ephippia.

### A.5.3 CULTURE METHODS FOR FATHEAD MINNOW (P. PROMELAS)

The culture methods for fathead minnow are adapted from Peltier and Weber (1985).

#### A.5.3.1 Distribution

The fathead minnow is widely distributed in North America, and is found in a wide range of habitats. This species is most abundant in brooks, small streams, creeks, ponds, and small lakes. It is tolerant of high temperature and turbidity, and low oxygen concentrations. The fathead minnow is primarily omnivorous; young fish have been reported to feed on organic detritus from bottom deposits, unicellular and filamentous algae, and planktonic organisms. Adults feed on aquatic insects, worms, small crustaceans, and other animals.

#### A.5.3.2 Life Cycle

The natural history and spawning behavior of the fathead minnow (Markus 1934, Flickinger 1973, Andrews and Flickinger 1974, and others) are well known. Male and female fathead minnows show sexual dimorphism at maturity. The breeding males develop a conspicuous, narrow, elongated, gray, fleshy pad of spongy tubercules on the back, anterior to the dorsal fin, and two or three rows of strong nuptial tubercules across the snout. The sides of the body of breeding males become almost black except for two wide vertical bars which are light in color. The females remain quite drab.

Spawning females release a small number of eggs (usually 100 to 150) at a time. The eggs are adhesive and attach to the under side of the spawning substrate. The females have an ovipositor (urogenital structure) to help deposit the eggs on the under side of objects. Flickinger (1966) indicated that the ovipositor is noticeable at least a month prior to spawning. The reported size of the eggs varies from 1.15 mm (Markus 1934) to 1.3 mm in diameter (Wynne-Edwards 1932).

Immediately after the eggs are laid, they are fertilized by the male, and the female is driven off. Once eggs are deposited in the nest, the male becomes very aggressive and will use the large tubercules on his snout to help drive off all intruding small fishes. In addition to fertilizing and guarding the eggs, the male agitates the water around the eggs, which ventilates them and keeps them free of detritus. Some males will spawn with a number of females on the same substrate, so that the nest may contain eggs in various stages of development. The number of eggs per nest will vary from as few as nine or 10 to as many as 12,000.

The ovaries of the females contain eggs in all stages of development. Females spawn repeatedly as the eggs mature. A female may deposit eggs in more than one nest. Although the average number of eggs per spawn per female is generally 100 to 150, large females may lay 400 to 500 eggs per spawn.

The hatching time depends on temperature. The average hatching time required at 25°C is 4.5 to 6 days. The newly hatched young (fry) are about 5 mm long, white in color, with large black eyes. In warm, food-rich water, growth is rapid. Adult size is probably not reached until the second year, and the males generally grow faster than the females. The fathead minnow is short-lived, and rarely survives to the third year.

### A.5.3.3 Taxonomy

The specific name (P. promelas) appears to be incorrectly applied to this fish because the fathead minnow does not fit the description originally given by Rafinesque (Lee et al. 1980). Also, some geographic variations have been noted in the morphology of the fathead minnow. Vandermeer (1966), in a statistical analysis of geographic variations in taxonomic characters, stated that subspecies intergrade clinally. The American Fisheries Society (1980), however, recognizes only one species.

### A.5.3.4 Morphology and Identification -- General Characters

Fathead minnows vary greatly in many characteristics throughout their wide geographic range. The morphology and characters for identification are taken from Trautman (1957), Clay (1962), Hubbs and Lagler (1964), Eddy and Hodson (1961), and Scott and Crossman (1973). They are small fish, being typically 43 mm to 102 mm, or an average of about 50 mm, in total length.

The mouth is terminal. The snout does not extend beyond the upper lip and is decidedly oblique. Nuptial tubercules, which occur on mature males only, are large and well-developed on the snout, and rarely extend beyond the nostrils. They occur in three main rows, with a few on the lower jaw. In addition to nuptial tubercules, there is an elongate fleshy or spongy pad extending in a narrow band from the nape to the dorsal fin. Wide anteriorly, the pad narrows to engulf the first dorsal ray.

A dark spot is usually present in front of the dorsal fin in mature males. Dymond (1926) and Trautman (1957) have described a saddle-like pattern, often associated with breeding males in which a light area develops just behind the head and another beneath the dorsal fin. Standard lengths are usually less than four and one-half times the body depth.

### A.5.3.5 Hybridization

Trautman (1957) stated that under some conditions the fathead minnow may hybridize with the bluntnose minnow, P. notatus (Rafinesque). He also indicated that fathead and bluntnose minnows were competitors, and that the fathead occurred in greater population densities only where the bluntnose was absent or comparatively few in number.

### A.5.3.6 Culturing Methods

#### A.5.3.6.1 Sources of Organisms--

When test fish are secured from an outside source, there is no guarantee that they will be of the age, size, quality, or condition needed for performing bioassays. Additionally, because of the possibility of occurrence of disease in fish brought into the laboratory, it may be necessary to give them a prophylactic treatment for disease to prevent and/or eliminate infections. Such treatment places a stress on the test fish and requires a quarantine period of seven days.

To avoid the problems associated with using fish of unknown condition and age, an in-house laboratory culture facility can be developed. Such a facility can provide a continuous supply of eggs (embryos) and/or other developmental stages of known age, which are free of disease or other stress, for use in toxicity tests.

#### A.5.3.6.2 Laboratory Culture Facility--

Fathead minnows can be cultured in either a static or flow-through system. Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture

facility consists of the following components: water supply, spawning tanks, holding tanks, egg incubation units, and rearing tanks.

1. Water Supply--Synthetic water or dechlorinated tap water can be used, but untreated well water is preferred. If a static system is used, it is necessary to equip each aquarium with a carbon filter system (similar to those sold for tropical fish hobbyists) to control the accumulation of metabolic wastes in the water.

   The dissolved oxygen concentration in the culture tanks should be maintained near the 100% saturation level, using air stones if necessary. Brungs (1971), in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all dissolved oxygen concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the reaeration of flowing water for culture systems.

   Adequate procedures for culture maintenance must be followed consistently to avoid poor water quality in the culture system. Tanks are cleaned monthly or more often as required. They should be kept free of debris, e.g., excess food, detritus, and waste, by daily siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks with a pipet. To avoid excessive buildup of algal growth, periodically scrape the walls of aquaria. Activated charcoal and filter pads in the aquaria filtration systems should be changed weekly. Culture water may be maintained by preparation of synthetic water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

2. Spawning Tanks--The spawning unit is designed to simulate conditions in nature conducive to spawning. Fathead minnows spawn in spring and summer, and the lab photoperiod is maintained to mimic the natural spawning conditions. For breeding tanks, it is convenient to use 76-L (20-gal) aquaria. Spawning tanks must be held at a temperature of 25 ± 2°C. Each aquarium is equipped with a heater, continuous filtering unit, and spawning substrates. The photoperiod for the spawning tanks must be rigidly controlled and maintained at 16 h light and 8 h dark (5:00 AM to 9:00 PM is a convenient photoperiod). An illumination level of 540 to 1080 lux is adequate. The breeding fish are fed all the frozen brine shrimp and tropical fish flake food or dry commercial fish food that they can eat twice daily (8:00 AM and 4:00 PM) during the week and once a day on weekends. If properly maintained, each breeding tank will produce a spawn of 100 to 200 eggs approximately every four days.

   It is necessary to provide a surface for the laying and fertilizing of eggs, as well as a territory for the male. The number of substrates placed in each tank depends on the size of the tank and the number of males. Two substrates are used for each male. The substrates should be placed equidistant from each other and staggered from each other on the bottom of the tanks so that the spawning territories of the males do not overlap. The recommended spawning substrates consist of inverted 15-cm (6-in.) sections of 10-cm (4-in) diameter clay tile or PVC cut in half longitudinally. Four substrates are used in each 76-L (20-gal) spawning tank.

   The number of fish in each aquarium, as well as the ratio of females to males, depends on the size of the aquarium. Five to eight females and two males are placed in each breeding tank. Spawning females must be replaced periodically (every two to three months) with other mature, egg-laden females to ensure a continuous supply of eggs.

3. Egg Incubation Units--Once spawning conditions are right, eggs are produced. Fathead minnows spawn in the early morning hours. They should not be disturbed except for a morning feeding (8:00 AM) and daily examination of substrates for eggs between 9:30 AM and 10:30 AM. In nature, the male protects, cleans, and aerates the eggs until they hatch. In

the laboratory, however, it is necessary to remove the eggs from the tanks, to prevent them from being eaten by the adults, for ease of handling when recording egg count and hatchability.

The substrates are each lifted carefully and checked for newly spawned eggs. If eggs are observed on a tile, remove the tile immediately from the spawning tank, stand it on end in a small (10- to 20-L) tank, and place an active air stone at the bottom of the tank near the egg mass. Vigorous aeration inhibits fungal growth better than weak aeration. Alternately, the eggs can be removed from the substrate with a razor blade or with a rolling action of the index finger, placed in a 1-L jar or beaker, and aerated with sufficient vigor to keep them in suspension.

If fungal growth is a problem, it can be controlled for the entire incubation period by adding 3 mL of 1% methylene blue stock solution per liter of water in the incubation vessels (see Herwig 1979, pp. 160-161).

During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Fungus should be removed quickly, and the substrates should be returned to the incubation tanks as rapidly as possible so that viable eggs are not damaged by desiccation.

Hatching takes four to five days at an optimal temperature of 25°C. Hatching can be delayed several (two to four) days by incubating at 10° to 15°C.

4. Rearing Tanks--Newly hatched fish are transferred daily from the egg incubation tanks to small (8-L) rearing tanks, using a large-bore pipet, until the hatch is complete. New rearing tanks are set up on a weekly basis to separate fish by age group. A density of 150 fry per liter is suitable for the first four weeks. The rearing tanks are allowed to follow ambient laboratory temperatures of 20° to 23°C, but sudden, extreme variations in temperatures must be avoided. Fathead minnow fry are fed freshly hatched brine shrimp (Artemia) nauplii twice daily until they are four weeks old. Utilization of older (larger) brine shrimp nauplii will result in starvation of the young fish because they are unable to ingest the larger food organisms. (see Section A.5.4, Culture Methods for Brine Shrimp, for instructions on the preparation of brine shrimp nauplii).

Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#1 and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate.

**A.5.3.6.3 Disease Control--**

Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. If disease occurs, treat with 0.6 mL Procaine Penicillin-G per 76-L tank. Hoffman and Mitchell (1980) have assembled a list of some chemicals that have been used commonly for fish diseases and pests.

In aquatic culture systems that use recycled water and filtration, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite, and nitrate concentrations (Collins et al. 1976). These changes could cause the death of the culture organisms.

Do not transfer equipment from one tank to another without first disinfecting it. If an outbreak of disease occurs, any equipment, such as nets, air lines, tanks, etc., that has been exposed to diseased fish should be disinfected with sodium hypochlorite.

### A.5.3.7 Test Organisms

Fish that are 1-90 days old are used in the toxicity test. It is recommended that fish 3-5 (± 12 h) old from at least three broods be used for testing. Before the fish are removed from the holding tank, most of the water in the holding tank should be siphoned off. The fish are then transferred to finger bowls, using a large-bore, fire-polished, glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb. It is important to note that larvae should not be handled with a dip net. Dipping small fish such as these with a net will result in very high mortality. The same large-bore, fire-polished, glass tube discussed above should be used to transfer the fish from the finger bowl to the test vessels. The fish are counted as they are caught and are placed gently into the test vessels.

## A.5.4 CULTURE METHODS FOR BRINE SHRIMP

The culture methods for brine shrimp are adapted from Peltier and Weber (1985).

### A.5.4.1 Sources of Brine Shrimp Eggs

Although there are many commercial sources of brine shrimp eggs, the Brazilian strain is preferred because it has low concentrations of chemical residues. One source is Aquarium Products, 180 L Penrod Ct, Glen Burnie, MD 21061. Reference Artemia cysts are available in limited quantity from the Artemia Reference Center, State University of Ghent, Belgium, J. Plafeousfruar 22, B 9000, Ghent, Belgium. In the United States, contact the Environmental Research Laboratory, EPA, South Ferry Road, Narragansett, RI 02882, for information on reference materials and commercial sources of good quality Artemia eggs.

### A.5.4.2 Incubation Chamber and Procedure

A 2000-mL separatory funnel makes a convenient brine shrimp hatching vessel. A satisfactory but less expensive apparatus can be prepared by cutting the bottom from a 2-L plastic soft drink bottle and inserting a rubber stopper with a flexible tube and pinch cock. Add approximately 1800 mL dechlorinated water and four tablespoons of non-iodized salt to the hatching vessel and shake until the table salt (NaCl) dissolves. Alternatively, synthetic or filtered natural seawater can be used. Add the desired quantity of eggs (usually 1.5 to 2.0 mL, dry) to the vessel and mix well. The quantity of eggs used depends on feeding requirements. For example, approximately 15 mL (dry) of eggs will provide enough brine shrimp nauplii to feed 1000 to 1500 newly hatched fish in four to six 8-L tanks.

After the appropriate volume of eggs is added to the hatching vessel, air is vigorously bubbled through a 1-mL pipet which is lowered through the neck of the funnel so that the tip rests at the bottom. Aeration will keep the eggs and newly hatched nauplii from settling on the bottom, where the dissolved oxygen quickly would be depleted.

The eggs will hatch in 24 h at a temperature of 27°C. Hatching time varies with incubation temperature and the geographic strain of Artemia used.

### A.5.4.3 Harvesting the Nauplii

The nauplii can be easily harvested in the following manner:

1. After 24 h at 27°C, remove the pipet supplying air and allow the nauplii to settle to the bottom of the separatory funnel. The empty egg shells will float to the top.

2. After approximately 5 min, using the stopcock of the separatory funnel, drain the nauplii into a 250-mL beaker.

3. After a second 5-min period and, again using the stopcock, drain any nauplii remaining in the separatory funnel into the beaker.

4. The nauplii are further concentrated by pouring the suspension into a small cylinder that has one end closed with #20 plankton netting.

5. The concentrate is resuspended in 50 mL of appropriate culture water, mixed well, and dispensed with a pipet.

6. Discard the remaining contents of the hatching vessel and wash the vessel with hot soap and water.

7. Prepare fresh salt water for each new hatch. To have a fresh supply of Artemia nauplii daily, several hatching vessels must be set up and harvested on alternate days.

## A.5.5 CULTURE METHODS FOR ALGAE, S. CAPRICORNUTUM

The culture methods for algae are adapted from Miller et al. (1978) and ASTM (1981).

### A.5.5.1 Test Organisms

S. capricornutum is a unicellular coccoid green alga. Section A.5.1 provides information on sources of "starter" cultures.

### A.5.5.2 Stock Algal Cultures

Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (see Section 5.5.3). The volume of stock culture initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125-mL flask to 2 L in a 4-L flask. The remainder of the starter culture can be held in reserve for up to six months in a refrigerator in the dark at 4°C.

Maintain the stock cultures at 24 ± 2°C, under continuous cool-white fluorescent lighting of 4300 ± 430 lux. Shake continuously at 100 cpm.

Transfer 1 to 2 mL of stock culture weekly to 1 L of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination.

To maintain unialgal culture material over a long period of time, it is advantageous to use a semi-solid medium containing 1.0% agar. The medium is placed in sterile petri dishes, and a 1-mL portion of a liquid algal culture is streaked onto it and incubated as described above. Place rubber

bands around the petri dishes to reduce evaporation loss of the medium. Fresh (liquid) stock cultures may be started at four-week intervals by transfer of cells from a single clone in a petri dish to an appropriate volume of liquid medium.

### A.5.5.3 Culture Medium

The culture medium is used to maintain the stock cultures of the test organisms. Culture medium is used as a control in each test and as the diluent in each test. Steps for preparing the culture medium follow:

1. Prepare five stock nutrient solutions using reagent-grade chemicals as described in Table A-2.

2. Add 1 mL of each stock solution, in the order listed in Table A-2, to approximately 900 mL of distilled or deionized water. Mix well after the addition of each solution.

3. Dilute to 1 L, mix well, and adjust the pH to 7.0 ± 0.1, using 0.1 N sodium hydroxide or hydrochloric acid, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table A-3.

4. Sterilize the filtration apparatus, including 0.2-µm pore diameter membranes. Membranes may be constructed of Teflon, glass fiber, or cellulose triacetate. Membranes must be autoclaved to ensure an aseptic system.

5. Immediately filter the pH-adjusted medium through a 0.2-µm pore diameter membrane at a vacuum of not more than 380 mm (15 in) mercury, or at a pressure of not more than one-half atmosphere (8 psi).

6. Place the filtered medium immediately into sterile culture flasks. No further sterilization steps are required before the inoculation of the medium.

Unused sterile medium should stored at 4°C in the dark for not more than one month before use. Storage may result in substantial loss of water by evaporation.

## A.5.6 CULTURE METHODS FOR THE EARTHWORMS (EISENIA FOETIDA)

### A.5.6.1 Introduction

Large numbers of E. foetida of known age and size can be easily cultured. Considerable information has been accumulated concerning their growth (Neuhauser et al. 1980), reproduction (Hartenstein et al. 1979), and physical requirements (Kaplan et al. 1980). This information and the ease of culturing makes E. foetida an appropriate earthworm for testing the toxicity of solid hazardous wastes.

### A.5.6.2 Distribution

E. foetida can be found worldwide in its specific habitat, which includes manure and soils with a high proportion of organic matter (Fender 1985).

### A.5.6.3 Life Cycle

E. foetida reaches maturity in seven to eight weeks at 22 ± 2°C. It is very prolific; a single worm produces two to five cocoons per week, each with several worms.

**TABLE A-2. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES**

| Nutrient Stock Solution | Compound | Amount Dissolved in 500 mL Distilled Water |
|---|---|---|
| 1 | $MgCl_2\ 6H_2O$ | 6.08 g |
| | $CaCl_2\ 2H_2O$ | 2.20 g |
| | $H_3BO_3$ | 92.8 mg |
| | $MnCl_2\ 4H_2O$ | 208.0 mg |
| | $ZnCl_2$ | 1.64 mg[a] |
| | $FeCl_3\ 6H_2O$ | 79.9 mg |
| | $CoCl_2\ 6H_2O$ | 0.714 mg[b] |
| | $Na_2MoO_4\ 2H_2O$ | 3.63 mg[c] |
| | $CuCl_2\ 2H_2O$ | 0.006 mg[d] |
| | $Na_2EDTA\ 2H_2O$ | 150.0 mg |
| | $NaNO_3$ | 12.750 g |
| 2 | $MgSO_4\ 7H_2O$ | 7.350 g |
| 3 | $K_2HPO_4$ | 0.522 g |
| 4 | $NaHCO_3$ | 7.50 g |

[a] $ZnCl_2$ -- Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[b] $CoCl_2\ 6H_2O$ -- Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[c] $Na_2MoO_4\ 2H_2O$ -- Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #1.

[d] $CuCl_2\ 2H_2O$ -- Weigh out 60.0 mg and dilute to 1000 mL. Dilute 1 mL of this solution to 10 mL. Add 1 mL of the second dilution to Stock #1.

**TABLE A-3. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM**

| Macronutrient Concentrations (mg/L) | | | |
|---|---|---|---|
| Compound | | Element | |
| $NaNO_3$ | 25.5 | N | 4.20 |
| $MgCl_2\ 6H_2O$ | 12.2 | Mg | 2.90 |
| $CaCl_2\ 2H_2O$ | 4.41 | Ca | 1.20 |
| $MgSO_4\ 7H_2O$ | 14.7 | S | 1.91 |
| $K_2HPO_4$ | 1.04 | P | 0.186 |
| | | K | 0.469 |
| $NaHCO_3$ | 15.0 | Na | 11.0 |
| | | C | 2.14 |

| Micronutrient Concentrations (ug/L) | | | |
|---|---|---|---|
| Compound | | Element | |
| $H_3BO_3$ | 185 | B | 32.5 |
| $MnCl_2\ 4H_2O$ | 416 | Mn | 115 |
| $ZnCl_2$ | 3.27 | Zn | 1.57 |
| $CoCl_2\ 6H_2O$ | 1.43 | Co | 0.354 |
| $CuCl_2\ 2H_2O$ | 0.012 | Cu | 0.004 |
| $Na_2MoO_4\ 2H_2O$ | 7.26 | Mo | 2.88 |
| $FeCl_3\ 6H_2O$ | 160 | Fe | 33.1 |
| $Na_2EDTA\ 2H_2O$ | 300 | -- | -- |

### A.5.6.4 Morphology and Taxonomy

Classification of the earthworm Eisenia foetida is controversial. Some authorities recognize two subspecies, foetida and andrei (Edwards 1984), while others recognize the subspecies as distinct species (Fender, 1985). Identification with the naked eye is not 100% accurate. There are no studies which indicate a sensitivity difference between andrei and foetida; conventional toxicological literature does not differentiate between the two. For these reasons, this manual simply refer to E. foetida as the designated name of the species used in the test. CERL has used andrei as its test organism for the last two years. In any case, results of the tests should include an identification of the organism used.

### A.5.6.5 Culture Methods

#### A.5.6.5.1 Sources of Organisms--

Starter cultures of E. foetida can be obtained from Mr. Julian Stewart, Vittor & Associates, 8100 Cottage Hill Road, Mobile, AL 36695.

#### A.5.6.5.2 Culture Vessels--

The earthworms can be grown in any number of suitable containers including glass, polyethylene, and wood. The size of the container affects the ability to handle and move the earthworms as required by the laboratory protocol. Excessive container weight may make moving, feeding, and harvesting procedures difficult. The containers should be covered with a glass lid containing air holes.

#### A.5.6.5.3 Culture Media--

The earthworms are grown in peat moss bedding. The bedding pH is adjusted between 5 and 8 by adding up to three percent by weight of $CaCO_3$. When using peat, care must be taken to avoid overwatering. Peat tends to become water logged with time and anaerobic conditions develop. Indications of anaerobic conditions occur after two to three months and include (1) a change in color between the bottom bedding material and the upper one to two inches of material, and (2) development of a strong odor.

#### A.5.6.5.4 Culture Conditions--

E. foetida stock cultures are maintained in a growth chamber in the dark at the same temperature as used for testing. The chambers must be capable of maintaining a temperature of $22 \pm 2°C$.

#### A.5.6.5.5 Feeding--

A mixture of alfalfa pellets is used as the maintenance organic food substrate for the earthworms. The mixture is saturated with water and aged for two weeks in a sealed container. The aged food is sprinkled on the surface of the culture tray. Alfalfa pellets can be obtained from agricultural feed and supply stores.

#### A.5.6.5.6 Culture Maintenance--

Crowding of adult worms in the growth container must be avoided. Crowding decreases the growth rate and reproduction efficiency. Splitting the bedding material in half every three to four months with pH-adjusted peat moss will prevent overcrowding (Neuhauser et al. 1980).

When the bedding appears dry on the surface, water should be added. However, saturation can cause the earthworms to crawl out of the rearing container. Therefore, water should be added at a rate that keeps the material wet, but avoids standing water in the bottom of the rearing pan. Water use will depend on the ambient temperature and relative humidity. Watering additions can, however, easily be adjusted to the particular conditions of the rearing room.

A pH range of 5 to 8 is best for optimum growing conditions. Earthworms subjected to moisture and pH conditions outside the optimum range will leave the container or die.

**A.5.6.5.7 Production Level--**

At least four culture containers should be maintained to ensure a sufficient number of earthworms on a continuing basis.

**A.5.6.6 Test Organisms**

Adult E. foetida (at least two months old with a clitellum) are used for the toxicity test. The earthworms should weigh 300 to 500 mg and be from the same culture container. From 150 to 300 earthworms may be required for each test.

## SECTION A.6

## DILUTION WATER

This section is adapted from Horning and Weber (1985).

Recommended dilution water is synthetic soft water, except for tests with D. magna, which require moderately hard water (see Section A.5.2) and for algal tests, which use algal growth medium. Soft dilution water can also be made by diluting well water or dechlorinated tap water (see below) with distilled or deionized water to obtain soft water with the same pH, hardness, and alkalinity as the synthetic soft water. The dilution water is considered acceptable if D. spp. show adequate survival, growth, and reproduction when cultured in the water. If possible, water should conform to ASTM specification D 1193, Type III (1981). Type III grade reagent water shall be prepared by distillation, ion exchange, reverse osmosis, or a combination thereof, followed by polishing with a 0.45 um membrane filter.

Dechlorinated water should be used as dilution water only as a last resort, because it is usually difficult to completely remove all the residual chlorine or chlorinated organics, which may be very toxic to the test organisms. Sodium thiosulfate is recommended for dechlorination (1.0 mg anhydrous sodium thiosulfate/L will reduce chlorine concentrations of 1.5 mg/L). After dechlorination, total residual chlorine must be non-detectable.

If it is necessary to pass the dilution water through a deionizer to remove unacceptably high concentrations of copper, lead, zinc, fluoride, or other toxic substances before use, it must be reconstituted to restore the calcium and magnesium removed by the deionization process.

To prepare synthetic fresh water, use the reagents listed in Table A-4. For example, to prepare 20 L of soft synthetic water, follow these steps:

1. Place 19 L of distilled or deionized water in a properly cleaned plastic carboy.

2. Add sufficient $MgSO_4$, $NaHCO_3$, and KCl to the plastic carboy, and stir well.

3. Add sufficient $CaSO_4\ 2H_2O$ to 1 L of distilled or deionized water in a separate flask, place on a magnetic stirrer until the calcium sulfate has dissolved, and add to the plastic carboy and stir well.

4. Aerate vigorously for 24 h (with air filtered through cotton to remove oil) to dissolve the added chemicals and stabilize the medium.

The measured pH, hardness, and alkalinity of the aerated water will approximate that indicated under "Final Water Quality" in Table A-4.

**TABLE A-4. PREPARATION OF SYNTHETIC FRESH WATER[a]**

| Water Type | Reagent Added (mg/L)[b] | | | | Final Water Quality | | |
|---|---|---|---|---|---|---|---|
| | $NaHCO_3$ | $CaSO_4.2H_2O$ | $MgSO_4$ | KCl | pH[c] | Hardness[d] | Alkalinity |
| Very Soft | 12.0 | 7.5 | 7.5 | 0.5 | 6.4-6.8 | 10-13 | 10-13 |
| Soft[e] | 48.0 | 30.0 | 30.0 | 2.0 | 7.2-7.6 | 40-48 | 30-35 |
| Moderately Hard[f] | 96.0 | 60.0 | 60.0 | 4.0 | 7.4-7.8 | 80-100 | 60-70 |
| Hard | 192.0 | 120.0 | 120.0 | 8.0 | 7.6-8.0 | 160-180 | 110-120 |
| Very Hard | 384.0 | 240.0 | 240.0 | 16.0 | 8.0-8.4 | 280-320 | 225-245 |

[a]Taken in part from Marking and Dawson (1973).
[b]Add reagent-grade chemicals to distilled or deionized water.
[c]Approximate equilibrium pH after 24 h of aeration.
[d]Expressed as mg $CaCO_3$/L.
[e]Recommended dilution for tests using species other than D. magna.
[f]Recommended for tests using D. magna.

## SECTION A.7

## HAZARDOUS WASTE SAMPLING AND HANDLING

In this section, procedures are described for sampling and handling hazardous wastes and for making the elutriates required for testing the toxicity of solid hazardous waste samples to aquatic and terrestrial test organisms.

### A.7.1 APPARATUS AND EQUIPMENT

1. Sample Containers

   a. 3-gal plastic pails with lids and handles
   Cardinal Plastics #384-P, or equivalent
   Akron, OH
   (206) 562-9600

   b. 5-gal steel paint cans with crimp lids
   Freund Can Company #1260-4450, or equivalent
   Chicago, IL
   1-800-621-2808

   c. 2.5-gal cubitainer
   VWR Scientific #243000-155, or equivalent
   P.O. Box 7900
   San Francisco, CA
   (415) 468-7150

   d. Plastic Trash Bags, 1 x 2'
   VWR Scientific #11215-392, or equivalent

   e. ORM-E Labels
   Labelmaster, Chicago, IL 60646
   (312) 478-0900

2. Flint-glass jars (1/2-gal or 2-L) with Teflon-lined lids for preparing elutriates.

3. Top-loading balance, 1- to 2000-g capacity

4. Crystallizing dishes, 100-mm diameter

5. Drying oven

6. End-over-end shaker
   Rota-Tox rotary extractor
   Associated Design and Manufacturing Co.
   Alexandria, VA

7. Glass beakers, 250 mL

8. Qualitative crepe filter paper, 185-mm diameter, coarse porosity

9. Glass chemical funnel, 100-mm (top inside diameter) x 95-mm (stem length)

10. 10-mL pipets

11. 500-mL glass Erlenmeyer flasks

12. Aluminum foil

13. Refrigerated centrifuge with 1-L or more capacity.

### A.7.2 HAZARDOUS WASTE SAMPLING, HANDLING, AND STORAGE

Proper collection, packaging, and shipping of hazardous waste site samples is critical. Proper sampling and shipping ensures sample integrity, safety in handling, and an adequate data base for sample processing and future sampling requirements. The following sections outline the minimum requirements for sampling and shipping hazardous wastes.

#### A.7.2.1 Aqueous Hazardous Wastes

From 3.7 to 7 L of hazardous waste solution are required to perform all six toxicity tests and to conduct routine chemical analyses (Table A-5). Except for the fathead minnow test, which requires the greatest volume of solution, the volumes in Table A-5 are greater than those actually required and make some allowance (100%) for wastage and repetition of tests.

All sample containers should be rinsed with sample water before being filled with sample.

Fill the 2.5-gal cubitainers with the aqueous sample. Seal the screw cap with tape. Place the cubitainer into two plastic bags. Seal the bags with tape and place the sealed sample into a 3-gal plastic pail, which is then sealed. Place the plastic pail into a 5-gal metal paint can and crimp the lid.

Complete the information on tape or label, which identifies the packager and date, using an indelible pen. Affix strips of tape over the crimped edges in at least two places.

Laboratory chain-of-custody procedures must always be followed when handling h[illegible]dous wastes.

#### A.7.2.2 Solid Hazardous Wastes

From 3.6 to 4.5 kg of solid hazardous waste is required to perform all six toxicity tests and to conduct routine chemical analyses (Table A-5). Except for the fathead minnow test, the volumes in Table A-5 are more than those actually required and make some allowance (100%) for waste and repetition of tests.

Line the 3-gal plastic pail with two plastic bags. Completely fill plastic bags inside the pail with waste material, then seal the plastic bags with tape. Secure lid on pail with tape and insert the pail into a plastic outer bag. Seal the outer bag with tape and insert soil or sediment sample into the 5-gal metal paint can. Crimp lid on can.

Complete the information on tape or label, which identifies the packager and date, using an indelible pen. Affix strips of tape over the crimped edges in at least two places.

Laboratory chain-of-custody procedures must always be followed when handling hazardous wastes.

**TABLE A-5. QUANTITIES OF AQUEOUS HAZARDOUS WASTE, SOLID HAZARDOUS WASTE, AND/OR ELUTRIATE REQUIRED TO PERFORM THE SIX TOXICITY TESTS AND ROUTINE CHEMICAL ANALYSES**

| Test | Aqueous or Solid Hazardous Waste | |
|---|---|---|
| | Aqueous Waste or Solid Elutriate Required (mL) | Sample Required (g, dry weight) |
| *D. spp* | 600 | 150 |
| Fathead Minnow | | |
| ≤ 5-d old | 1200 | 300 |
| > 5-d old | 4500 | 1125 |
| Algae | 600 | 150 |
| Lettuce root elongation | 240 | 60 |
| Earthworm | none | 1500 |
| Lettuce seed germination | none | 1200 |
| Chemical Analyses | 1000 | 250 |
| Totals | | |
| Fathead minnows ≤ 5-d old | 3640 | 3610 |
| > 5-d old | 6940 | 4435 |

### A.7.2.3 Labeling Requirements

All containers will be identified according to the labeling requirements discussed below. A data sheet (Figure A-2) must be filled out for each sample with as much detail as possible.

U.S. Department of Transportation (DOT) regulations require that environmental samples collected from hazardous materials disposal sites are labeled as **Other Regulated Materials, "E" Class (ORM-E)**. This label will be affixed to the lid and the bucket of all collected environmental samples. This label identifies the sample as being potentially hazardous, flammable, corrosive, poisonous, etc., but containing less than a reportable quantity of the sample. All such labels should be clearly identifiable (white on black or vice versa) and affixed with permanent ink or paint. A highlighted border of at least 1 in. is required. If sample contents are known, or if reportable quantities of various substances (corrosive, poison, etc.) are contained or anticipated in the sample, then labeling must comply with DOT CFR-49 specifications. These specifications are found in Section 172 of the DOT Hazardous Materials Shipping and Handling Regulations. These regulations can be found at the office of any carrier authorized to haul hazardous materials.

### A.7.3 SAMPLE HANDLING AND PRESERVATION

Aeration during collection and transfer of solid and aqueous hazardous wastes should be minimized to reduce the loss of volatile chemicals. The time elapsed from collection of a sample to the initiation of the toxicity tests should not exceed 72 h. Sample toxicity may be affected when held for longer than 72 h. Samples must be chilled after collection and maintained at 4°C until used for testing, unless toxicity tests are initiated within 24 h of sample collection.

Samples collected for on-site tests should be used within 24 h. Samples collected for off-site toxicity testing are to be chilled to 4°C when collected, shipped on ice to the central laboratory, and transferred to a refrigerator (4°C) until start of test. Every effort must be made to initiate the test with a sample on the day of its arrival in the laboratory.

### A.7.4 AQUEOUS SAMPLE PREPARATION

The aqueous hazardous wastes must be filtered through a (30-μm) plankton net to remove indigenous organisms that may attack the test organisms. Waters used in algal toxicity tests must be filtered through a 0.45-μm pore diameter filter before use. It may be necessary to first coarse-filter the waste water through a nylon sieve having 2-mm holes to remove debris and/or break up large, floating or suspended solids.

The dissolved oxygen (DO) concentration in the dilution water should be near saturation before use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH.

If the dilution water and waste water must be warmed to bring them to the prescribed test temperature, supersaturation of dissolved gases may become a problem. To prevent this problem, the waste and dilution waters are heated to 25°C and checked for DO with a probe. If the DO exceeds 100% saturation, the solutions are aerated vigorously with an air stone (usually 1 to 2 min) until the DO is lowered to 100% saturation.

### A.7.5 ELUTRIATE PREPARATION

From 3.7 to 7 L of elutriate are required to perform all six toxicity tests and to conduct routine chemical analyses (Table A-5). The volume of elutriate prepared must be sufficient for all test concentrations and for chemical analyses. Except for the fathead minnow test, the volumes in Table A-5 are more than those actually required and make some allowance (100%) for wastage and

HMAT Sample Data Sheets

1. Date of Collection

2. Name of Sample Site

3. Location of Site

4. Site Sequential Sample Number

5. Type of Sample _____ Soil
_____ Water
_____ Groundwater
_____ Other

6. Quantity Processed _____ Soil
_____ Water
_____ Groundwater
_____ Other

7. Septh of Sample (m) _____ (cm) _____

8. Date Received at CERL _____

9. Chemical Constituents

Known _____
_____
_____
_____
_____

Suspected _____
_____
_____
_____
_____

10. Collected by: Name _____
Address _____
Phone _____

11. Notes _____
_____
_____
_____
_____
_____

Figure A-2. Waste site sample collection data sheets.

repetition of tests. Standard procedures for effluents and surface waters do not recommend filtering the elutriate. During the last 2 years, in cooperation with EPA's Environmental Response Team, CERL has filtered elutriate, surface waters, and ground waters. This was done for the specific purpose of assessing solute toxicity. If samples are filtered, then interpretation of results should recognize the potential for altered toxicity (see Section 1.4 and Section 7.7). If the sample is not filtered and suspended solids remaining in the waste water cause a physical stress to the D. spp. and fathead minnows, then sample filtration is required to remove the solids (see Sections A.8.2.11.8 and A.8.3.11.6).

The elutriates are prepared by adding deionized water to the waste equal to four times the dry weight of the sample. For the following calculation assume 1 mL of water weighs 1 g. Since most wastes will already contain some water, this residual water must be factored into the calculation of the amount of deionized water to be added. Waste samples should not be dried before preparing elutriates.

First, to determine the moisture content of the sample, place 125 g of sample into a clean crystallizing dish and weigh.

The combined weight of the dish and sample equals the initial wet weight. Dry the sample at 100 ± 5°C for 24 h. Cool in a desiccator and reweigh the dish and sample. The combined weight (to nearest gram) of the dish and dried sample equals the final dry weight. Save the dried soil in an air-tight container for use in determining the sample water holding capacity. The moisture fraction of the sample is calculated as follows:

$$\text{Moisture Fraction (MF)} = \frac{\text{initial wet weight (g)} - \text{final dry weight (g)}}{\text{subsample weight (125 g)}}$$

Each 2-L jar will hold about 1500 mL of water plus 375 g of dry sample (375 x 4 = 1500 mL). Three to five jars will be required to prepare enough elutriate for all tests. Calculate the volume of deionized water to be added to each jar as follows:

$$\text{Water Added to Elutriate Jar (mL)} = [1500\ \text{mL}] - [\text{moisture fraction} \times 375\ \text{g dry sample}]$$

Calculate the total wet weight of sample to add to the elutriate jar:

$$\text{Wet Sample Weight (g)} = [375\ \text{g dry sample}] + [\text{moisture fraction} \times 375\ \text{g dry sample}]$$

Add the correct amount of wet sample to each elutriate jar and then add the correct amount of deionized water.

Mix the jar contents (end-over-end) in the dark at 20 ± 2°C for 48 h.

Centrifuge the suspension at 10,000 rpm for 10 min at 4°C. Combine and thoroughly mix the elutriates from each elutriate jar. Do not concentrate the elutriates. It is best to use the elutriates for toxicity testing immediately after preparation. If they are not used immediately, store them at 4°C in the dark and use them within 72 h. Refer to Section A.7.4 for sample filtration requirements.

## A.7.6 WATER HOLDING CAPACITY

The earthworm and lettuce seed germination toxicity tests require that the moisture content of the test soils be determined. Water holding capacities of both the artificial and site soil must be known so appropriate moisture adjustments can be made.

Place 100 g of dried sample (as measured by the procedure in Section A.7.5) into a 250-mL glass beaker. Add 100 mL of deionized water to the sample. Mix thoroughly with a glass stir rod to ensure that all sample particles are wetted and that a slurry of sample and water exists.

Next, fold a circle of 185-mm diameter, coarse porosity, qualitative crepe filter paper into quarters and place in a 100-mm (top inside diameter) by 95-mm (stem length) glass funnel. The folded filter paper should be level with the top of the glass funnel. Slowly add 9 mL of deionized water, using a pipet, to the filter paper to wet the entire surface. Measure the combined weight of the funnel and hydrated filter paper. Add the weight of the dried soil (100 g) to the weight of the funnel and hydrated filter paper to obtain the initial weight.

Place the funnel in a 500-mL glass Erlenmeyer flask. Slowly pour the slurry of soil and water onto the hydrated filter paper held in the funnel. Rinse any soil remaining on the beaker and stir rod into the funnel with deionized water. Use the minimum volume of water necessary to ensure that all of the solid material has been washed onto the filter. Cover the funnel tightly with aluminum foil and allow it to drain for 3 h at room temperature.

Weigh the funnel, hydrated filter paper, and soil to obtain the final weight. The water holding capacity, expressed as mL water/100 g soil, equals the difference between the final and initial weights of the funnel, filter, and sample.

The water holding capacity of the artificial soils used in the lettuce seed germination and earthworm test must also be determined, as above. If the recipe is not changed, the water holding capacity of the artificial soil should not change.

## A.7.7 CHEMICAL OR PHYSICAL MODIFICATION OF TEST MATERIALS

For the purpose of detecting and quantifying, toxicity tests should be run as described in the appendix. However, some modifications or adjustments may be desirable to answer site specific toxicological questions. If such adjustments or modifications are made, they may modify the toxicities of the solutions.

For example, increasing test solution (or soil) pH, which will also increase the alkalinity and hardness (if a calcium or magnesium compound is used), will significantly affect the toxicities of most metals by increasing complexation with carbonates and bicarbonates and decreasing metal solubilities and toxicities. Increasing the calcium concentration will generally decrease metal uptake and toxicity. Increasing the pH will also (1) increase the toxicities of ammonia and cyanide, (2) decrease the toxicity of hydrogen sulfide, (3) alter the toxicities of polar organic chemicals and (4) reduce the toxicities of acids. Decreasing the pH will generally have opposite effects on toxicity as those for increasing the pH.

Filtering will remove particulate matter, some of which may contribute to the toxicity of the entire mixture, plus some toxic chemicals in solution may be adsorbed by the filter. Thus, filtering may reduce the toxicities of some test solutions.

Modifying the chemical and physical properties of the test solutions may lead to underestimating the toxicities of some solutions or lead to identifying some toxic solutions as non-toxic (false negatives). If such modifications are made, it would be desirable to run parallel tests on unaltered test materials, so that the effects of these modifications can be quantified. In any case, such modifications should only be done with consideration of their potential effects on the toxicities of the test materials.

## SECTION A.8

## TOXICITY TEST METHODS

### A.8.1 INTRODUCTION

In this section, methods for the six toxicity tests are described. Section A.8.2 presents the methods for the D. pulex and D. magna toxicity test; Section A.8.3, the fathead minnow survival test; Section A.8.4, the algal growth test; Section A.8.5, the earthworm survival test; Section A.8.6, the lettuce seed germination test; and Section A.8.7, the lettuce root elongation test. For each toxicity test, the scope and application, apparatus and equipment, reagents and consumable materials, and procedures, as well as other useful information, are provided.

### A.8.2 DAPHNIA PULEX AND DAPHNIA MAGNA TOXICITY TEST

#### A.8.2.1 Scope and Application

This method (adapted from Peltier and Weber [1985], Horning and Weber [1985], and ASTM [1980]) measures the acute toxicity of hazardous waste solutions to the cladocerans, D. pulex and D. magna, during a 48-hour static exposure. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the test organisms. This method should be performed by, or under the supervision of, professionals experienced in aquatic toxicity testing.

Detection limits of the toxicity of a hazardous waste solution or pure substance are organism dependent.

#### A.8.2.2 Summary of Method

The test species, D. pulex or D. magna, is exposed in a static system for 48 h to different concentrations of hazardous waste solutions. Test results are based on survival.

#### A.8.2.3 Definitions

For definitions of key terms, refer to the Glossary.

#### A.8.2.4 Interferences

Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section A.4, Facilities and Equipment). Adverse effects of low dissolved oxygen (DO) concentrations or high concentrations of suspended and/or dissolved solids may mask the presence of toxic substances. Pathogenic organisms in the dilution water and test solution may affect test organism survival and confound test results.

Improper hazardous waste handling and elutriate preparation may also adversely affect test results (see Section A.7, Hazardous Waste Sampling and Handling).

#### A.8.2.5 Safety

For a discussion on safety, see Section A.2, Health and Safety.

### A.8.2.6 Apparatus and Equipment

1. Laboratory D. spp. culture unit -- See Section A.5.2. This test requires at least 180 2- to 25-hour-old D. spp. neonates.

2. Sample containers -- for sample shipment and storage, see Section A.7, Hazardous Waste Sampling and Handling.

3. Environmental chamber, incubator, or equivalent facility with temperature control (20 ± 2°C).

4. Water purification system -- Millipore Milli-Q or equivalent that produces Type II ASTM water.

5. Test vessels -- 100-mL borosilicate glass beakers. Three beakers are required for each test concentration. The beakers should be covered with glass covers during the test.

6. Racks for test vessels -- Plexiglass racks drilled to hold 10 test vessels each.

7. Dissecting microscope -- for examining organisms in the test chambers.

8. Light box -- for illuminating organisms during examination.

9. Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-mL, for culture work and preparation of test solutions.

10. Volumetric pipets -- Class A, 1- to 100-mL.

11. Serological pipets -- 1- to 10-mL, graduated.

12. Pipet bulbs and fillers -- Propipet$^{R}$ or equivalent.

13. Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, 5 mm ID -- for transferring organisms.

14. Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

15. Glass or electronic thermometers -- for measuring water temperatures.

16. Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

17. National Bureau of Standards certified thermometer -- see EPA Method 170.1, EPA 1979b.

18. pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

19. Miscellaneous apparatus and equipment, and transfer containers should be constructed of materials as indicated in Section A.4, Facilities and Equipment.

### A.8.2.7 Reagents and Consumable Materials

1. Reagent water -- defined as activated-carbon-filtered distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 4 above).

2. Dilution water -- see Section A.6, Dilution Water. Soft dilution water (40 to 48 mg/L as $CaCO_3$) is recommended for tests with D. pulex, and moderately hard water (80 to 100 mg/L as $CaCO_3$) is recommended for tests with D. magna.

3. Hazardous waste solutions -- see Section A.7, Hazardous Waste Sampling and Handling.

4. Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, EPA 1979b).

5. pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

6. Membranes and filling solutions for DO probe (see EPA Method 360.1, EPA 1979b), and reagents for modified Winkler analysis.

7. Laboratory quality assurance samples and standards for the above methods.

8. Specific conductivity standards (see EPA Method 120.1, EPA 1979b).

9. Reference toxicant solutions (see Section A.3, Quality Assurance).

10. Test organisms -- D. pulex or D. magna 2- to 24-hour-old neonates. (See information on culturing methods in Section A.5.2). D. magna or D. pulex can be used as the test species if the lowest concentration of test solution has a hardness value $\geq$80 mg/L as $CaCO_3$. Only D. pulex should be used as the test species if the lowest concentration of test solution has a hardness value $<$80 mg/L as $CaCO_3$. Use of D. magna in soft water solutions may lead to mortality caused by osmotic stress.

    a. The test organism (species being used) cultures should be started at least two weeks before the brood animals are needed, to provide an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolificacy.

    b. D. spp. may be shipped or otherwise transported in polyethylene bottles. A low density population (20-30 animals) will live as long as one week in a 1-L bottle filled three-fourths full with culture medium containing the trout chow diet (see Section A.5.2). Animals received from an outside source should be transferred to new culture medium gradually, over a period of 1 to 2 days, to avoid mass mortality.

    c. Culture -- See Section A.5.2 for D. spp. culture methods.

### A.8.2.8 Sample Collection, Preservation, and Handling

For a discussion on sample collection, preservation, and handling, see Section A.7, Hazardous Waste Sampling and Handling.

### A.8.2.9 Calibration and Standardization

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

**A.8.2.10 Quality Control**

For a discussion on quality control, see Section A.3, Quality Assurance.

**A.8.2.11 Procedure**

**A.8.2.11.1 Test Solutions--**

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

The volume of hazardous waste solution required for three replicates per concentration, each containing 50 mL of test solution, is approximately 300 mL. Enough test solution (approximately 700 mL) should be prepared at each concentration to provide 400 mL additional volume for chemical analyses.

**A.8.2.11.2 Hardness of Test Solutions--**

Measure the hardness of the lowest concentration of hazardous waste solution to be tested. The hardness of the aqueous hazardous waste or elutriate must be measured before the test is initiated to determine if D. magna or D. pulex is the appropriate species for the test.

D. magna or D. pulex can be used as the test species if the lowest concentration of test solution has a hardness value $\geq$80 mg/L as $CaCO_3$. Only D. pulex should be used as the test species if the lowest concentration of test solution has a hardness value $<$80 mg/L as $CaCO_3$.

**A.8.2.11.3 Obtaining Neonates for the Test--**

This test method requires 2- to 24-hour-old neonates to begin the test. (See Section A.5.2, D. spp. Culture Methods.)

**A.8.2.11.4 Start of the Test--**

Measure the DO concentration of the aqueous sample or elutriate and dilution water. If the DO in the test solution and/or dilution water is low ($<$60% saturation), aerate before preparing the test solutions. Although aeration may be necessary, a loss of toxicity may occur due to volatilization or chemical interactions.

The test should begin as soon as possible, preferably within 24 h of sample collection. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to 20 ± 2°C and maintained at that temperature until portions are added to the dilution water.

Begin the test by randomly placing one neonate in each test beaker until 10 neonates are in each beaker. Because of their small size and difficulty in handling, the test chambers are usually placed in racks, 10 to a rack. The position of the test chambers is randomized in the racks at the beginning of the test, and on following days, the positions of the racks are randomized.

**A.8.2.11.5 Light, Photoperiod, and Temperature--**

The light quality and intensity should be at ambient laboratory levels, approximately 540 to 1080 lux, with a photoperiod of 16 h of light and 8 h of darkness. The test water temperature should be maintained at 20 ± 2°C.

**A.8.2.11.6 Feeding--**

The D. spp. are not fed during the test.

**A.8.2.11.7 Routine Chemical and Physical Analyses--**

At a minimum the following measurements are made:

- o DO, pH, conductivity, alkalinity and hardness are measured at the beginning and end of the test in a control beaker and in one test beaker at each test concentration.

- o Temperature is measured at the beginning of each 24-h exposure period in a control beaker and at each test concentration.

**A.8.2.11.8 Observations During the Test--**

Protect the D. spp. from unnecessary disturbance during the test. Make sure the D. spp. remain immersed during the performance of the above operations.

The D. spp. are best counted with the naked eye. The organisms are more easily seen if viewed against a black background. If counts are made without the aid of a stereo microscope, place the test vessels on a black strip of tape on a light box.

The numbers of live and dead D. spp. in each test chamber are recorded every 24 ± 2 h, and the dead D. spp. are discarded. Death is indicated by lack of movement of the body or appendages when gently prodded. Unusual behavior such as lethargy, floating on the surface, and unusual phenomena, such as the presence of surface films, precipitates, or organisms caught in particulate matter, should be noted and recorded.

**A.8.2.11.9 Termination of the Test--**

The test is terminated after 48 h of exposure, when the numbers of live and dead D. spp. are recorded.

**A.8.2.11.10 Acceptability of Test Results--**

For the test results to be acceptable, mean survival in the controls must be at least 90%.

**A.8.2.11.11 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-6.

**A.8.2.12 Calculations**

See Section 2 of the main text for data analysis methods.

The effect measured during the toxicity tests using D. spp. is death.

Report the LC50 and its 95% confidence limits. The LC50 is an estimate of the median lethal concentration.

**A.8.2.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes precision of the test; the accuracy of toxicity tests cannot be determined.

**TABLE A-6. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR D. PULEX AND D. MAGNA TOXICITY TEST**

| | | |
|---|---|---|
| 1. | Test type: | Static |
| 2. | Temperature (°C): | 20 ± 2°C |
| 3. | Light quality: | Ambient laboratory light |
| 4. | Light intensity: | 540 to 1080 lux (ambient laboratory levels) |
| 5. | Photoperiod: | 16 h light, 8 h dark |
| 6. | Test vessel size: | 100 mL |
| 7. | Test solution volume: | 50 mL |
| 8. | Age of test organisms: | 2 - 24 h |
| 9. | Number of test organisms per chamber: | 10 |
| 10. | Number of replicate chambers per dilution: | 3 |
| 11. | Feeding regime: | Do not feed |
| 12. | Aeration: | None |
| 13. | Dilution water: | D. pulex - 40 to 48 mg/L $CaCO_3$<br>D. magna 80 to 100 mg/L $CaCO_3$ |
| 14. | Site sample hardness: | <80 mg/L $CaCO_3$ D. magna<br>>80 mg/L $CaCO_3$ D. pulex |
| 15. | Dilution factor: | 0.5 |
| 16. | Test duration: | 48 h |
| 17. | pH range: | ≥6 - ≤10 |
| 18. | Effect measured: | Death |

* If pH is outside this range, results may reflect pH toxicity. Adjustments of pH to either 6 or 10 may result in altered toxicity to other constituents (see Section 1.4 and Section A.7.7).

## A.8.3 FATHEAD MINNOW TOXICITY TEST (PIMEPHALES PROMELAS)

### A.8.3.1 Scope and Application

This method (adapted from Peltier and Weber [1985], Horning and Weber [1985], and ASTM [1980]) estimates the acute toxicity of hazardous waste solutions to the fathead minnow (P. promelas), using fish 3 to 5 days old in a static test. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. This method should be performed by, or under the supervision of, professionals experienced in aquatic toxicity testing.

Detection limits of the toxicity of a hazardous waste solution or pure substance are organism dependent.

### A.8.3.2 Summary of Method

Fathead minnows 3 to 5 days old are exposed in a static system for 48 h to different concentrations of hazardous waste solutions. Test results are based on the survival of the fish.

### A.8.3.3 Definitions

For the definitions of key terms, refer to the Glossary.

### A.8.3.4 Interferences

Toxic substances may be introduced by contaminants in or on dilution water, glassware, sample hardware, and testing equipment (see Section A.4, Facilities and Equipment). Adverse effects of low dissolved oxygen (DO) concentration or high concentrations of suspended and/or dissolved solids may mask the presence of toxic substances. Improper hazardous waste handling and elutriate preparation may adversely affect test results (see Section A.7, Hazardous Waste Sampling and Handling).

Pathogenic organisms in solutions may affect test organism survival, and also confound test results.

To avoid further interferences, the fish should not be fed during the test.

### A.8.3.5 Safety

For a discussion on safety, see Section A.2, Health and Safety.

### A.8.3.6 Apparatus and Equipment

1. Fathead minnow and brine shrimp culture units -- see Sections A.5.3 and A.5.4. This test requires 150-300 fish, one to 90 days old. It is preferable to obtain fish from an in-house fathead minnow culture unit. Fish 3-5 days old (± 12 h) are strongly recommended for testing. If fish less than five days old are used, smaller test vessels (150-mL) with a smaller volume (100 mL) of test solution can be used. Using fish less than five days old would reduce the volume of test solution required from 4500 mL to 1200 mL. If it is not feasible to culture fish in-house, fish can be shipped in well-oxygenated water in insulated containers. All fish used in a test should be ± 1 days in age and should come from a pool of fish consisting of at least three separate spawnings.

2. Sample containers -- for sample shipment and storage (see Section 4, Facilities and Equipment).

3. Environmental chamber or equivalent facility with temperature control (20 ± 2°C).

4. Water purification system -- Millipore Milli-Q or equivalent.

5. Test chambers -- borosilicate glass or non-toxic disposable plastic labware. If fish ≤ 5 days old are used, 150-mL beakers are appropriate. If older fish are used, 1-L beakers are appropriate. At least three beakers are required for each concentration and control. To avoid potential contamination from the air, the chambers should be covered during the test.

6. Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-mL for making test solutions.

7. Volumetric pipets -- Class A, 1- to 100-mL.

8. Serological pipets -- 1- to 10-mL, graduated.

9. Pipet bulbs and fillers -- Propipet$^{R}$ or equivalent.

10. Droppers and glass tubing with fire-polished edges, 4 mm ID - - for transferring small fish.

11. Small dip nets for transferring larger fish.

12. Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

13. Glass or electronic thermometers -- for measuring water temperatures.

14. Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

15. National Bureau of Standards certified thermometer (see EPA Method 170.1, EPA 1979b).

16. pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

17. Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section A.4, Facilities and Equipment.

**A.8.3.7 Reagents and Consumable Materials**

1. Reagent water -- defined as activated-carbon-filtered distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 4 above)

2. Aqueous hazardous wastes or a hazardous waste elutriate -- see Section A.7, Hazardous Waste Sampling and Handling.

3. Dilution water -- see Section A.6, Dilution Water.

4. Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, EPA 1979b).

5. pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

6. Membranes and filling solutions for DO probe (see EPA Method 360.1, EPA 1979b), or reagents for modified Winkler analysis.

7. Laboratory quality assurance samples and standards for the above methods.

8. Specific conductivity standards (see EPA Method 120.1, EPA 1979b).

9. Reference toxicant solutions (see Section A.3, Quality Assurance).

10. Test organisms -- Fathead minnows one to 90 days old. Fish three to five days old ( ± 12 h) are strongly recommended. (see Section A.5.3, Culture of Fathead Minnows).

**A.8.3.8 Sample Collection, Preservation, and Storage**

For a discussion on collecting, preserving, and storing samples, see Section A.7, Hazardous Waste Sampling and Handling.

**A.8.3.9 Calibration and Standardization**

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

**A.8.3.10 Quality Control**

For a discussion on quality control, see Section A.3, Quality Assurance.

**A.8.3.11 Procedures**

**A.8.3.11.1 Test Solutions--**

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision ( ± 300%). A dilution factor of 0.5 provides greater precision ( ± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

If the test solution is known or suspected to be highly toxic, a lower range of concentrations should be used, beginning at 10%. If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of concentrations can be added.

For fish $\leq$5 days old, the total volume of solution required for three replicates per concentration, each containing 100 mL of test solution, is approximately 600 mL. For older fish, the volume of solution required for three replicates per concentration, each containing 750 mL of test solution, is approximately 4500 mL. Enough test solution at each concentration should be prepared to provide 400 mL additional volume for chemical analyses.

**A.8.3.11.2 Start of the Test--**

Measure the DO concentration of the aqueous sample or elutriate and dilution water. If the DO in the test solution and/or dilution water is low (60% saturation), aerate before preparing the test solutions. Although aeration may be necessary, a loss of toxicity may occur due to volatilization or chemical interactions.

Tests should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to 20 ± 2°C and maintained at that temperature until portions are added to the dilution water.

Regardless of the size or age of fish used in a test, the loading of fish per chamber must not exceed 0.8 g/L. Weigh a subsample of fish to be used in the test to determine their average weight and the appropriate loading rate.

The test is initiated by placing fish, one or two at a time, into each test chamber, until each chamber contains up to 10 fish, for a total of at least 30 fish for each concentration.

Randomize the position of test chambers at the beginning of the test. The amount of water added to the chambers when transferring the fish to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

**A.8.3.11.3 Light, Photoperiod, and Temperature--**

The light quality and intensity should be at ambient laboratory levels, which is approximately 540 to 1080 lux, with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 20 ± 2°C.

**A.8.3.11.4 Feeding--**

As mentioned previously, the fish are not fed during the test.

**A.8.3.11.5 Routine Chemical and Physical Analyses--**

At a minimum, the following measurements are made:

- DO and temperature are measured at the beginning of each 24-h exposure period and at the end of the exposure period in a control beaker and in one test beaker at each test concentration.
- Temperature, pH, conductivity, alkalinity, and hardness are measured at the beginning and end of the test in a control beaker and at each test concentration.

**A.8.3.11.6 Observations During the Test--**

Protect the fish from unnecessary disturbance during the test. Make sure the fish remain immersed during the performance of the above operations.

The numbers of live and dead fish in each test chamber are counted and recorded 24 ± 2 h after the test is initiated, and the dead fish are discarded. Death is indicated by lack of movement of the body or appendages when gently prodded. Unusual behavior, such as lethargy, floating on the

surface, and unusual phenomena, such as the presence of surface films, precipitates, and organisms caught in particulate matter, should be noted and recorded.

**A.8.3.11.7 Termination of the Test--**

The test is terminated after 48 h of exposure. At termination, the numbers of live and dead fish in each test chamber are counted.

**A.8.3.11.8 Acceptability of Test Results--**

For the test results to be acceptable, mean survival in the controls must be at least 90%.

**A.8.3.11.9 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-7.

**A.8.3.12 Calculations**

The effect measured during the toxicity tests using fathead minnow is death.

See Section 2 of the main text for data analysis methods.

Report the LC50 and its 95% confidence limits. The LC50 is an estimate of the median lethal concentration.

**A.8.3.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes precision of the test; the accuracy of toxicity tests cannot be determined.

**TABLE A-7. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (P. PROMELAS) TOXICITY TEST**

| | | |
|---|---|---|
| 1. | Test type: | Static |
| 2. | Temperature (°C): | 20 ± 2°C |
| 3. | Light quality: | Ambient laboratory light |
| 4. | Light intensity: | 540-1080 lux (ambient laboratory levels) |
| 5. | Photoperiod: | 16 h light, 8 h dark |
| 6. | Test chamber size: | 150-mL or 1-L |
| 7. | Test solution volume: | 100 or 750 mL/replicate |
| 8. | Age of test organisms: | 3-5 days |
| 9. | Number of test organisms per chamber: | 10 fish/chamber; not to exceed 0.8 g/L. Minimum of 30 fish/test concentration. |
| 10. | Number of replicate chambers per dilution: | Minimum of 3 |
| 11. | Feeding regime: | Do not feed |
| 12. | Aeration: | If the DO concentration falls below 40% saturation, gently aerate solutions. |
| 13. | Dilution water: | Same as culture water |
| 14. | Test concentrations: | At least 5 and a control |
| 15. | Dilution factor: | 0.5 |
| 16. | Test duration: | 48 h |
| 17. | Effect measured: | Death |

## A.8.4 ALGAL GROWTH (SELENASTRUM CAPRICORNUTUM)

### A.8.4.1 Scope and Application

Unicellular algae are important producers of oxygen and form the basis of the food web in aquatic ecosystems. Algal species and communities are sensitive to environmental changes and their growth may be inhibited or stimulated by the presence of pollutants.

The method (Porcella 1983, Horning and Weber 1985) measure the toxicity of hazardous waste solutions to the fresh water alga, S. capricornutum, during a four-day, static exposure. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the test organisms.

Detection limits of the toxicity of a hazardous waste solution or pure substance are organism dependent.

### A.8.4.2 Summary of Method

A Selenastrum population is exposed in a static system to a series of concentrations of hazardous waste solutions for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, or chlorophyll content.

### A.8.4.3 Definitions

For the definitions of key terms, refer to the Glossary.

### A.8.4.4 Interferences

Toxic substances may be introduced by contaminants in or on dilution water, glassware, sample hardware, and testing equipment (see Section A.4, Facilities and Equipment). In addition, high concentrations of suspended and/or dissolved solids may mask the presence of toxic substances. Improper hazardous waste sampling and elutriate preparation may also adversely affect test results (see Section A.7, Hazardous Waste Sampling and Handling). The EDTA present in the stock culture medium used for dilution water may chelate some toxic metals potentially present in hazardous waste solutions. This may lead to the underestimation of the toxicities of these solutions. Pathogenic and/or predatory organisms in the dilution water and test solutions may affect test organism survival, thereby confounding test results. The amount of natural nutrients present in the test solutions or dilution water may also affect test results. The pH of test solutions should fall between 5 to 10; values outside this range can inhibit growth and confound test results (Miller et al. 1978).

### A.8.4.5 Safety

For a discussion on safety, see Section A.2, Safety and Health.

### A.8.4.6 Apparatus and Equipment

1. Laboratory Selenastrum culture unit -- see Section A.5.5. To test solution toxicity, sufficient numbers of log-phase- growth organisms must be available.

2. Sample containers -- for sample shipment and storage, see Section A.7, Hazardous Waste Sampling and Handling.

3. Environmental chamber, incubator, or equivalent facility-- with cool-white fluorescent illumination (4300 ± 430 lux) and temperature control (24 ± 2°C).

4. Mechanical shaker -- Capable of providing orbital motion at the rate of 100 cycles per minute (cpm).

5. Light meter -- with a range of 0-11,000 lux.

6. Water purification system -- Millipore Milli-Q or equivalent.

7. Balance, analytical -- capable of accurately weighing 0.0001 g.

8. Reference weights, Class S -- for checking performance of balance.

9. Glass or electronic thermometers -- for measuring water temperatures.

10. Bulb-thermometer or electronic-chart type thermometers -- for continuously recording temperature.

11. National Bureau of Standards certified thermometer (see EPA Method 170.1, EPA 1979b).

12. Meters: pH and specific conductivity -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

13. Fluorometer (optional) -- Equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

14. Cuvettes for spectrophotometer -- 1- to 5-cm light path.

15. Electronic particle counter (optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

16. Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condenser, and light source (inverted or conventional microscope).

17. Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

18. Centrifuge -- with swing-out buckets having a capacity of 15 to 100 mL.

19. Centrifuge tubes -- 15- to 100-mL, screw-cap.

20. Filtering apparatus -- for membrane and/or glass fiber filters.

21. Volumetric flasks and graduated cylinders -- Class A, 10- to 1000-mL, borosilicate glass, for culture work and preparation of test solutions.

22. Volumetric pipets -- Class A, 1- to 100-mL.

23. Serological pipets -- 1- to 10-mL, graduated.

24. Pipet bulbs and filters -- Propipet$^{R}$ or equivalent.

25. Wash bottles -- for rinsing small glassware, instrument electrodes, and probes.

26. Culture flasks -- 1- to 4-L borosilicate, Erlenmeyer flasks.

27. Test flasks -- 125- or 250-mL borosilicate, Erlenmeyer flasks.

28. Preparation of glassware -- prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes, and vials used in algal bioassays as follows:

    a. Wash with non-phosphate detergent solution, preferably heated to 50°C or hotter. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher is highly recommended.

    b. Rinse thoroughly with tap water and drain well.

    c. All new test flasks and all flasks that, through use, may become contaminated with toxic organic substances, must be rinsed with acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, proceed with the next step.

    d. If acetone is used in Step c, rinse thoroughly with tap water. If the heat treatment is used, go directly to Step e.

    e. Carefully rinse with a 10% solution (by volume) of reagent-grade hydrochloric acid (HCl); fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.

    f. Rinse with tap water and drain well.

    g. To neutralize any residual acid, rinse with a saturated solution of $Na_2CO_3$.

    h. Rinse five times with tap water and then five times with deionized or distilled water.

    i. Dry in an oven, cover the mouth of each vessel with aluminum foil or other closure, as appropriate, before storing.

29. Use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

A.8.4.7 Reagents and Consumable Materials

1. Reagent water -- defined as carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 7 above).

2. Test solution and dilution water -- see Section A.6, Dilution Water, and Section A.7, Hazardous Waste Sampling and Handling. The dilution water is algal medium (see Section A.5.5).

3. Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, EPA 1979b).

4. pH buffers 4, 7, 8 and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

5. Laboratory quality assurance samples and standards for the above methods.

6. Specific conductivity standards (see EPA Method 120.1, EPA 1979b).

7. Reference toxicant solutions (see Section A.3, Quality Assurance).

8. Acetone -- pesticide quality or equivalent.

9. Dilute hydrochloric (or nitric) acid -- carefully add 10 mL of concentrated HCl (or $HNO_3$) to 90 mL of reagent water.

10. Test Organisms -- log-phase-growth S. capricornutum. See Section A.5.5 for culturing methods.

**A.8.4.8 Sample Collection, Preservation, and Handling**

For a discussion on collecting, preserving, and handling, see Section A.7, Hazardous Waste Sampling and Handling.

**A.8.4.9 Calibration and Standardization**

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

**A.8.4.10 Quality Control**

For a discussion on quality control, see Section A.3, Quality Assurance.

**A.8.4.11 Procedures**

**A.8.4.11.1 Test Solutions--**

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

If the test solution is known or suspected to be highly toxic, a lower range of concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%).

The volume of test solution required for the test is 0.5 or 1 L depending on whether 125 mL or 250 mL beakers respectively are used. To reduce the volume of hazardous water solution required, it is recommended that the 125 ml flask with 50 mL of test solution be used. Prepare enough test solution at each test concentration (approximately 550 mL or 700 mL) to provide 50 mL or 100 mL of test solution for each of three replicate test chambers and 400 mL for chemical analyses. Dilution water consists of stock culture medium.

Test solutions may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution is added per liter of test solution before preparing the test dilutions. Thus, all test treatments and controls will contain at least the basic amount of nutrients.

The amount of nutrients present in the test dilutions may effect test results (Section A.8.4.4), however, ASTM is still considering the proportional amendment method (Porcella 1983). In the proportional amendment method, full strength algal assay medium (AAM) is used to dilute site samples for the test series. Dilution of the site sample with AAM should insure adequate nutrients for the algae. An 80 percent test solution (20 percent AAM), however, is the maximum permissible test concentration which ensures the presence of adequate nutrients. AAM nutrients are not spiked into the test solution prior to its dilution with algal assay medium. The different concentrations of AAM in the test series is accounted for in the formula for calculating percent effect (Section 2.1.2).

If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the test solution and dilution water must be filtered through a GF/A, GF/C, or equivalent pore diameter filter, and checked for "background" particle count before it is used in the test. Note: Filtration may strip volatile substances from the solution, decreasing its toxicity.

If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

**A.8.4.11.2 Preparation of Inoculum--**

The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using S. capricornutum harvested from a four- to seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of 10,000 cells/mL in the test flasks. Assuming the use of 125-mL flasks, each containing 50 mL of test solution, the inoculum must contain 500,000 cells/mL. Assuming the use of 250-mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL. The following steps provide an example of how to estimate the volume of stock culture required to prepare the inoculum:

If the four- to seven-day stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 25 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 25,000,000 cells or 12.5 mL of stock solution (25,000,000/2,000,000) to provide sufficient inoculum. It is advisable to use a volume 20% to 50% in excess of the minimum volume required to cover accidental loss in transfer and handling.

1. Determine the density of cells (cells/mL) in the stock culture (for this example, assume 2,000,000 per mL).

2. Calculate the required volume of stock cultures as follows:

$$= \frac{\text{No. of test flasks} \times \text{Vol. of test solution/flask} \times 10^4 \text{ cells/mL}}{\text{Cell density (cells/mL) in stock culture}}$$

$$= \frac{25 \text{ flasks} \times 100 \text{ mL/flask} \times 10^4 \text{ cells/mL}}{2 \times 10^6 \text{ cells/mL}}$$

$$= 12.5 \text{ mL stock culture}$$

[Note: cell density is determined according to procedures described below.]

3. Add 0.5 mL of stock culture to each flask.

4. Mix well and determine the cell density in the makeup water. Some cells will be lost in the concentration process. Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.

**A.8.4.11.3 Start of the Test--**

Tests should begin as soon as possible, preferably within 24 h of test solution preparation. If the persistence of test solution sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after test solution preparation. Just prior to testing, the temperature of the sample should be adjusted to 24 ± 2°C and maintained at that temperature until the sample is diluted for testing.

The test begins when the algae are added to the test flasks. Mix the inoculum well, and add 1 mL to the test solution in each flask.

**A.8.4.11.4 Light, Photoperiod, and Temperature--**

Test flasks are incubated under continuous illumination at 4300 ± 430 lux at 24 ± 2°C, and should be shaken continuously at 100 cpm on a mechanical shaker. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. This step may be omitted if it can be verified that test specifications are met at all positions.

**A.8.4.11.5 Routine Chemical and Physical Analyses--**

At a minimum, the following measurements are made:

- Alkalinity, hardness, pH, and conductivity are measured in extra control solutions and extra test solutions (see Section A.8.7.11.1) at the beginning of the test and in a control flask and one test flask at each test concentration at the end of the test.

- Temperature is measured at the beginning of each 24-h exposure period in a control flask and at each test concentration.

**A.8.4.11.6 Observations During the Test--**

Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one to two days in the test. It is recommended, therefore, to determine the algal growth response daily, as described below.

**A.8.4.11.7 Termination of the Test--**

The test is terminated 96 h after initiation. It is recommended the algal growth in each flask is measured by cell counts. If there interferences on the particle counter, then the chlorophyll content can be measured to determine the reliability of the particle counter. Regardless of the method used to monitor growth, the algae in the test solutions should be checked under the microscope to detect abnormalities in cell size or shape.

1. Cell counts--Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in $\mu m^3$/cell. The Coulter Counter is widely used and is discussed in detail by Miller et al. (1978).

If biomass data are desired for algal growth potential measurements, a Model ZBI or ZB Coulter Counter is used. However, the instrument must be calibrated with a reference sample of cells of known volume.

When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is suspended in a 1% sodium chloride electrolyte (such as Isoton$^R$), in a ratio of 1 mL of test culture to 9 mL (or to 19 mL) of 0.22-µm filtered saline solution (dilution of 10:1 or 20:1). The resulting dilution is counted using an aperture tube with a 100-µm diameter aperture. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume.

The following procedure is used:

a. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat this the two-step process at least once.

b. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.

c. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter Isoton$^R$).

d. Determine the cell density (and MCV, if desired).

As alternative methods, cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, or inverted microscope. For details on microscope counting methods, see APHA (1985) and Weber (1973). Whenever feasible, 400 cells per replicate are counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

2. **Chlorophyll Content**--Chlorophyll may be measured in vivo fluorometrically, or in vitro either fluorometrically or spectrophotometrically. In vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al. 1982).

The measurements are made as follows:

a. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of test solution filtered through a 0.45-µm particle retention filter.

b. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 4 mL of culture from the flask with a sterile point.

c. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min.)

d. Discard the sample.

**A.8.4.11.8 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-8.

**A.8.4.11.9 Acceptability of Test Results--**

The test results are acceptable if the algal cell density in the control flasks exceeds $10^6$ cells/mL at the end of the test and does not vary more than 10% among replicates.

**A.8.4.12 Calculations**

The effect measured during the toxicity tests is inhibition of growth. If the growth in any treatment is greater than the growth of controls, it is considered non-toxic.

See Section 2 in the main text for data analysis methods.

Report the EC50 and its 95% confidence limits. The EC50 is an estimate of the median effective concentration.

**A.8.4.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes precision of the test; the accuracy of toxicity tests cannot be determined.

**TABLE A-8. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE ALGAL GROWTH TEST (S. CAPRICORNUTUM)**

| | | |
|---|---|---|
| 1. | Test type: | Static |
| 2. | Temperature (°C): | 24 ± 2°C |
| 3. | Light quality: | "Cool white" fluorescent light |
| 4. | Light intensity: | 4300 ± 430 lux |
| 5. | Photoperiod: | Continuous illumination |
| 6. | Test flask size: | 125 mL or 250 mL |
| 7. | Test solution volume: | 50 mL or 100 mL |
| 8. | pH range: | $\geq$5 but $\leq$10 |
| 9. | Age of stock culture used for inoculum: | 4 - 5 days |
| 10. | Initial cell density: | 10,000 cells/mL |
| 11. | Number of replicate chambers per dilution: | 3 |
| 12. | Shaking rate: | 100 cpm continuous |
| 13. | Dilution water: | Algal stock culture medium |
| 14. | Dilution factor: | 0.5 |
| 15. | Test duration: | 96 h |
| 16. | Effect measured: | Growth inhibition (cell counts, chlorophyll content, biomass |

* If pH is outside this range, results may reflect pH toxicity. Adjustment of pH to 5.0 or 10.0 may result in altered toxicity of other constituents (see Section 1.4 and Section A.7.7)

## A.8.5 EARTHWORM SURVIVAL (EISENIA FOETIDA)

### A.8.5.1 Scope and Application

This method (modified from the methods described by Edwards [1984] and Goats and Edwards [1982]) estimates the acute toxicity of solid hazardous wastes to the earthworm (E. foetida) in a 14-d static test. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the test organisms. The method uses soil as the exposure medium because the exposure conditions closely resemble natural conditions. The method could also be used to test aqueous samples by using 100% artificial soil and by using the aqueous samples in place of distilled or deionized water to hydrate the test soils. This method should be performed by, or under the supervision of, professionals experienced in environmental toxicity testing.

Detection limits of the toxicity of an hazardous waste solution or pure substance are organism dependent.

### A.8.5.2 Summary of Method

Earthworms (E. foetida) are exposed in a static system for 14 days to different concentrations of solid hazardous wastes mixed with artificial soil. Test results are based on survival of the worms.

### A.8.5.3 Definitions

For definitions of key terms, refer to the Glossary.

### A.8.5.4 Interferences

Toxic substances may be introduced by contaminants in water, glassware, sample hardware, artificial soil, and testing equipment (see Section A.4, Facilities and Equipment). Low dissolved oxygen (DO) concentrations or saturation of soils with water may mask the presence of toxic substances.

Pathogenic organisms in test materials may also affect test organism survival, and confound test results.

Improper hazardous waste sampling and handling may adversely affect test results (see Section A.7, Hazardous Waste Sampling and Handling).

### A.8.5.5 Safety

For a discussion on safety, see Section A.2, Health and Safety.

### A.8.5.6 Apparatus and Equipment

1. Earthworm culture units -- see Section A.5.6. This test requires 150-300 earthworms >60 days old weighing 300 to 500 mg. It is preferable to obtain the earthworms from an in-house culture unit.

2. Sample containers -- for sample shipment and storage (see Section A.7, Hazardous Waste Sampling and Handling).

3. Environmental chamber or equivalent facility with temperature control (22 ± 2°C).

4. Water purification system -- Millipore Milli-Q or equivalent.

5. Balance, analytical -- capable of accurately weighing earthworms to 0.001 g.

6. Balance, top-loading -- capable of weighing soil samples to 1.0 g.

7. Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus worms or soil.

8. Test chambers -- standard 1-pint canning jars with screw-top lids and rings.

9. Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-mL.

10. Volumetric pipets -- Class A, 1- to 100-mL.

11. Serological pipets -- 1- to 10-mL, graduated

12. Pipet bulbs and fillers -- Propipet$^{R}$ or equivalent.

13. Bulb-thermograph or electronic-chart type thermometers--for continuously recording temperature.

14. National Bureau of Standards certified thermometer (see EPA Method 170.1, EPA 1979b).

A.8.5.7 Reagents and Consumable Materials

1. Reagent water -- defined as activated-carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 4 above).

2. Solid hazardous waste sample -- see Section A.7, Hazardous Waste Sampling and Handling.

3. Artificial soil consisting of (by weight) 10% 2.36-mm-screened sphagnum peat, 20% colloidal kaolinite clay, and 70% grade 70 silica sand. The artificial soil must be well mixed after it is prepared. Peat, sand, and clay can be obtained from local suppliers.

4. pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

5. Laboratory quality assurance samples and standards for the above methods.

6. Reference toxicant (see Section A.3, Quality Assurance).

7. Test organisms -- earthworms (E. foetida); see Section A.5.6 for culture methods for earthworms. The species taxonomy should be verified using appropriate systematic keys (Fender 1985). Adult E. foetida (at least two months old with a clitellum) used for each test should weigh 300 to 500 mg and be from the same culture container.

A.8.5.8 Sample Collection, Preservation, and Storage

For a discussion on collecting, preserving, and storing samples, see Section A.7, Hazard Waste Sampling and Handling.

### A.8.5.9 Calibration and Standardization

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

### A.8.5.10 Quality Control

For a discussion on quality control, see Section A.3, Quality Assurance.

### A.8.5.11 Procedures

#### A.8.5.11.1 Test Soils--

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

If the hazardous waste is known or suspected to be highly toxic, a lower range of concentrations should be used (such as 10%, 5%, 2.5%, 1.25%, and 0.63%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of concentrations should be added.

The volume of hazardous waste required for three replicates per concentration, each containing 200 g of test soil, is approximately 1200 g. Prepare enough test soil at each concentration to provide sufficient soil for chemical analyses.

#### A.8.5.11.2 Glassware Preparation--

Drill or punch a 1/16-in. hole into the center of each canning jar lid. Clean all jars using the procedures outlined in Section A.4, Facilities and Equipment. The rinse cycle in some dishwashers is colder than the wash cycle; this creates a thermal shock, which may crack the soft glass jars. The cleaned jars should be dried at 50°C in a forced air oven and stored with lids (without holes) on in a dust-free environment until needed. The lids should be examined after each use because the seal may deteriorate during repeated washing. Replace the lids and screw rings as needed.

#### A.8.5.11.3 Start of the test--

Tests should begin as soon as possible, preferably within 24 h of test soil collection. If the persistence of sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after test soil collection. Just prior to testing, the temperature of the test soils should be adjusted to 20 ± 2°C.

Homogenize the solid waste material using a blender. Mix the homogenized solid waste material with artificial soil to prepare 700 g each of a geometric series of test soil concentrations, e.g., 100, 50, 25, 12.5, 6.25, 3.13% dry weight/dry weight, plus controls (100% artificial soil). To ensure even distribution of the test soil mixture, the total amount for each concentration is mixed together in a blender before dividing into replicates.

After mixing, test soils need to be hydrated with deionized water to create a moist, but not saturated, testing environment. The earthworm test soils are to be hydrated to 75% of water holding capacity. Hydration water required to achieve the desired hydration is calculated as follows:

Hydration Water to be added (mL/100g) = $THW_{ts}$ - $EHW_{ts}$

$THW_{ts}$ (total test soil hydration water desired, mL/100 g) =
PHYD x [(PAS X $WHC_{as}$) + (?WS x $WHC_{ws}$)],

$EHW_{ts}$ (existing test soil hydration water, mL/100 g) =
[(PAS x $MF_{as}$) + (PWS x $MF_{ws}$)] x 100,

where PHYD = proportion of hydration required (e.g., 0.75);
PAS = proportion of artificial soil in test soil (e.g., 0.50);
$WHC_{as}$ = water holding capacity of the artificial soil in mL/100 g;
PWS = proportion of waste sample in the test soil;
$WHC_{ws}$ = the water holding capacity of the waste sample in mL/100 g;
$MF_{as}$ = moisture fraction of the artificial soil; and
$MF_{ws}$ = moisture fraction of the waste sample.

Ten earthworms are placed into each of the three replicate jars each containing 200 g (dry weight) of test soil. (Randomize the position of test chambers at the beginning of the test.) Adult earthworms should be handled with a spatula.

The earthworms should be placed on the surface of the test soil in a pint jar, capped, and secured. The tests are incubated to give a soil temperature of 20 ± 2°C under continuous light. The test jars must not be shaded.

**A.8.5.11.4 Light, Photoperiod, and Temperature--**

Lighting should be at continuous ambient laboratory levels, which is approximately (540 to 1080 lux), with no shading.

**A.8.5.11.5 Feeding--**

To avoid further interferences, the worms are not fed during the test.

**A.8.5.11.6 Routine Chemical and Physical Analyses--**

At a minimum, the following measurements are made:

- The pH of the test soils should be measured at the beginning and end of the test.
- The temperature of the environmental control chamber should be continuously monitored.
- The TOC of the test soils should be measured in one test jar at all test concentrations and in the control.

**A.8.5.11.7 Observations During the Test--**

Mortality is assessed by emptying the test soil onto a tray and sorting the worms from the soil. Dead worms are discarded. (E. foetida are considered dead when they do not respond to a gentle touch to their anterior.) Live worms are placed back into their test jars and placed on the surface of the soil. The numbers of live and dead worms in each test chamber are recorded at 14 days and the dead worms are discarded.

Decay of dead worms can be rapid and, if all 10 worms per container are not found, it can be assumed that the worms decayed beyond recognition.

**A.8.5.11.8 Termination of the Test--**

The test is terminated after 14 days of exposure. At termination, the number of live and dead worms in each test chamber are counted.

**A.8.5.11.9 Acceptability of Test Results--**

For the test results to be acceptable, mean survival in the controls must be at least 90%.

**A.8.5.11.10 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-9.

**A.8.5.12 Calculations**

The effect measured during the toxicity tests using E. foetida is death.

See Section 2 in the main text for a description of data analysis methods. Both 7-d and 14-d LC50s and 95% confidence intervals should be calculated.

**A.8.5.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes test precision; the accuracy of toxicity tests cannot be determined.

**TABLE A-9. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR E. FOETIDA SURVIVAL TEST**

| | |
|---|---|
| 1. Test type: | Static |
| 2. Soil temperature (°C): | 20 ± 2°C |
| 3. Light quality: | Ambient laboratory light |
| 4. Light intensity: | 540-1080 lux |
| 5. Photoperiod: | Continuous illumination |
| 6. Test vessel type and size: | 1-pint glass canning jars with rings and lids 1/8 inch air hole |
| 7. Test soil mass: | 200 g |
| 8. Test soil pH* | $\geq$ 4 but $\leq$ 10 |
| 9. Artificial soil (% weight): | 10% 2.36 mm-screened sphagnum peat, 20% colloidal kaolinite clay, and 70%-grade 70 silica sand |
| 10. Test soil moisture content: | 75% of water holding capacity |
| 11. Renewal of test materials: | None |
| 12. Age of test organisms: | >60 days |
| 13. Number of test organisms per chamber: | 10 |
| 14. Number of replicate chambers per dilution: | 3 |
| 15. Feeding regime: | Do not feed |
| 16. Dilution factor: | 0.5 |
| 17. Test duration: | 14 days |
| 18. Effect measured: | Death |

* If pH is outside this range, results may reflect ph toxicity. Adjustments of ph to 4.0 or 10.0 may result in altered toxicity of other constituents. See Sec 1.4. and A 7.7.

## A.8.6 LETTUCE SEED GERMINATION (LACTUCA SATIVA)

### A.8.6.1 Scope and Application

This method (modified from the method described by Thomas and Cline [1985]) estimates the acute toxicity of solid hazardous wastes to lettuce (L. sativa) in a 120-h static test. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the test organisms. The method uses soil as the exposure medium because the exposure conditions closely resemble natural conditions. The method could also be used to test aqueous samples by using 100% artificial soil and using the aqueous samples in place of distilled or deionized water to hydrate the test soils. This method should be performed by, or under the supervision of, professionals experienced in environmental toxicity testing.

Detection limits of the toxicity of a hazardous waste solution or pure substance are organism dependent.

### A.8.6.2 Summary of Method

Lettuce seeds (L. sativa) are exposed in a static system for 120 h to different concentrations of solid hazardous wastes mixed with artificial soil. Test results are based on the successful germination of the seeds.

### A.8.6.3 Definitions

For definitions of key terms, refer to the Glossary.

### A.8.6.4 Interferences

Toxic substances may be introduced by contaminants in water, glassware, sample hardware, artificial soil, and testing equipment (see Section A.4, Facilities and Equipment).

Improper hazardous waste sampling and handling may adversely affect test results.

Pathogenic organisms in test materials may affect test organism survival, and also confound test results.

### A.8.6.5 Safety

For a discussion on safety, see Section A.2, Health and Safety.

### A.8.6.6 Apparatus and Equipment

1. Wire mesh screens for sizing seeds (fractions of an inch): 1/6 x 1/28, 1/6 x 1/30, 1/6 x 1/32, 1/6 x 1/3, A.T. Ferrell and Company, Saginaw, MI 48601 or Seedburo Equipment Company, Chicago, IL 60607.

2. Forceps

3. pH meter

4. Storage bottles

5. 12" x 12" polyethylene resealable bags (e.g., Ziploc$^{R}$)

6. An illuminated magnifier

7. Sample containers -- for sample shipment and storage (see Section A.7, Hazardous Waste Sampling and Handling).

8. Controlled environmental chamber capable of maintaining a uniform temperature of 24 ± 2°C and 4300 ± 430 lux of light operating on a clock timer to control diurnal cycling.

9. Water purification system -- Millipore Milli-Q or equivalent.

10. Balance, top loading - capable of weighing soil samples to 0.1 g.

11. Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus samples.

12. Test chambers -- the bottom halves of plastic petri dishes, 150-mm wide by 15-mm high placed in 12" x 12" polyethylene resealable bags.

13. Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-mL.

14. Volumetric pipets -- Class A, 1- to 100-mL.

15. Serological pipets -- 1- to 10-mL, graduated.

16. Pipet bulbs and fillers -- Propipet$^{R}$ or equivalent.

17. Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

18. National Bureau of Standards certified thermometer (see EPA Method 170.1, EPA 1979b).

A.8.6.7 Reagents and Consumable Materials

1. Reagent water -- defined as activated-carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 9 above).

2. Solid hazardous waste sample

3. Artificial soil -- the artificial soil used in the lettuce seed germination test is commercially available, 20-mesh, washed silica sand.

4. Cover sand -- commercially available 16-mesh sand, which has been passed through a 20-mesh sieve to remove fines.

5. pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

6. Laboratory quality assurance samples and standards for the above methods.

7. Reference toxicant (see Section A.3, Quality Assurance).

8. Test organisms -- lettuce (butter crunch variety), Lactuca sativa L. The seeds used in this test are available from commercial seed companies. Seed from one lot should be purchased in amounts adequate for one year's testing. Information on seed lot, year, or growing season in which they were collected and germination percentage should be provided by the seed source. Only untreated (not treated with fungicide, repellents, etc.) seeds are acceptable for use in this toxicity test.

**A.8.6.8 Sample Collection, Preservation, and Storage**

For a discussion on collecting, preserving, and storing samples, see Section A.7, Hazardous Waste Sampling and Handling.

**A.8.6.9 Calibration and Standardization**

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

**A.8.6.10 Quality Control**

For a discussion on quality control, see Section A.3, Quality Assurance.

**A.8.6.11 Procedures**

**A.8.6.11.1 Test Soils--**

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

**A.8.6.11.2 Glassware Preparation--**

All glassware used in preparing the soil samples must be thoroughly washed as described in Section A.4, Facilities and Equipment.

**A.8.6.11.3 Start of the Test--**

Sample preparation should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of sample toxicity is not known, the maximum holding time should not exceed 36 h. Just prior to testing, the temperature of the test soils should be adjusted to 24 ± 2°C.

Homogenize the solid waste material using a blender. Mix the homogenized solid waste material with artificial soil to prepare 400 g each of a geometric series of test soil concentrations, i.e., 100, 50, 25, 12.5, 6.25, 3.13% dry weight hazardous waste/dry weight artificial soil, plus controls (100% artificial soil). To ensure even distribution of the test soil mixture, the total amount for each concentration is mixed together in a blender before dividing into replicates.

After purchase, size grading of seeds is carried out on the entire seed lot. Small samples, 100-150, are sized at a time.

In addition, the seed lot must be inspected. Trash, empty hulls, and damaged seed are removed.

Nest the four wire mesh sizing screens, with the one containing the largest holes on top and those with successively smaller holes in sequence below. A blank or bottom pan is used to collect the fraction that passes through all screens.

Pour the seeds onto the top screen, then shake the whole set of nested screens (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are set aside and retained. This procedure is repeated until all the seed in the lot is sized. The size class containing the most seed is selected and used for the duration of the tests. The seeds in each size class are divided into small lots, placed in separate envelopes or sacks, and stored in airtight, waterproof containers in a refrigerator at 4°C.

Place 100 g of each concentration of air-dried control or test soil in each of three replicate 150-mm plastic petri dishes. Randomize the position of the test containers. Place 40 seeds in each dish. Space the seeds at least 0.5 inch from the edge of the petri dish to ensure that they will be covered with test soil. Press the seeds into the test soil with the bottom of a clean beaker. Pour 90 g of cover sand on top of the hydrated test soil. Level the cover sand with a ruler or small piece of cardboard. Place petri dish cover over the bottom to prevent water loss and spillage prior to incubation.

Hydrate the test soils with deionized water to create a moist, but not saturated, testing environment. The lettuce seed germination test soils are to be hydrated to 85% of water holding capacity. Hydration water required to achieve the desired hydration is calculated as follows.

Hydration Water to be added (mL/100g) = $THW_{ts} - EHW_{ts}$

$THW_{ts}$ (total test soil hydration water desired, mL/100 g) =
$PHYD \times [(PAS \times WHC_{as}) + (PWS \times WHC_{ws})]$,

$EHW_{ts}$ (existing test soil hydration water, mL/100 g) =
$[(PAS \times MF_{as}) + (PWS \times MF_{ws})] \times 100$,

where PHYD = proportion of hydration required (e.g., 0.85);
PAS = proportion of artificial soil in test soil (e.g., 0.50);
$WHC_{as}$ = water holding capacity of the artificial soil in mL/100 g;
PWS = proportion of waste sample in the test soil;
$WHC_{ws}$ = the water holding capacity of the waste sample in mL/100 g;
$MF_{as}$ = moisture fraction of the artificial soil; and
$MF_{ws}$ = moisture fraction of the waste sample.

If $MF_{as} = 0$, which would be the case if the artificial soils are prepared from dry ingredients, then $EHW_{ts} = PWS \times MF_{ws} \times 100$.

Place each covered petri dish into a resealable polyethylene bag, centered over the bottom of the bag. Raise the sides of the bag into a vertical position over the dish. Remove the dish cover and seal (airtight) the bag, leaving as much air space as possible. Randomly distribute the bags in an environmental chamber.

**A.8.6.11.4 Light, Photoperiod, and Temperature--**

Incubate at 24 ± 2°C in the dark for 48 h, followed by 16 h of light and 8 h dark until termination of the test at the end of 120 h. Light intensity should be 4300 ± 430 lux.

**A.8.6.11.5 Routine Chemical and Physical Analyses--**

At a minimum, the following measurements are made:

- The pH of the test soils should be measured at the beginning and end of the test (Black 1965, 1982).
- Soil temperature is measured at the beginning of each 24-h exposure period in one replicate of each test concentration and in the control.

**A.8.6.11.6 Observations During the Test--**

No observations are made during the test.

**A.8.6.11.7 Termination of the Test--**

After 120 h, the number of germinated seeds in each dish is determined by counting each seedling that protrudes above the soil surface.

**A.8.6.11.8 Acceptability of Test Results--**

For the test results to be acceptable, mean germination in the controls must be at least 90%.

**A.8.6.11.9 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-10.

**A.8.6.12 Calculations**

The effect measured during the toxicity tests using lettuce (*L. sativa*) seeds is germination.

See Section 2 in the main text for a description of data analysis methods.

Report the LC50 and its 95% confidence limits. The LC50 is an estimate of the median lethal concentration.

**A.8.6.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes test precision; the accuracy of toxicity tests cannot be determined.

**TABLE A-10. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR LETTUCE SEED (L. SATIVA) GERMINATION TEST**

| | | |
|---|---|---|
| 1. | Test type: | Static |
| 2. | Temperature (°C): | 24 ± 2°C |
| 3. | Light quality: | Fluorescent |
| 4. | Light intensity: | 4300 ± 430 lux |
| 5. | Photoperiod: | Initial 48 h dark, followed by 16 h of light and 8 h of dark until termination of the test. |
| 6. | Test vessel type and size: | The bottom halves of plastic petri dishes, 150-mm wide by 15-mm high, placed in 12" x 12" polyethylene resealable bags |
| 7. | Test soil mass: | 100 g |
| 8. | Test soil moisture content: | 85% of water holding capacity |
| 9. | Artificial soil: | 20-mesh, washed silica sand |
| 10. | Test soil pH: | $\geq$4 but $\leq$10 |
| 11. | Renewal of test materials: | None |
| 12. | Age of test organisms: | seeds |
| 13. | Number of test organisms per chamber: | 40 |
| 14. | Number of replicate chambers per dilution: | 3 |
| 15. | Dilution factor: | 0.5 |
| 16. | Test duration: | 120 h |
| 17. | Effect measured: | Germination |

* If pH is outside this range, results may reflect pH toxicity. Adjustment of pH to 4.0 or 10.0 may result in altered toxicity to other constituents. See Sec 1.4 and A.7.7.

## A.8.7 LETTUCE ROOT ELONGATION (LACTUCA SATIVA)

### A.8.7.1 Scope and Application

This method (as modified from the method described by Porcella [1983] and Ratsch [1983]) estimates the acute toxicity of aqueous hazardous wastes and hazardous waste elutriates to lettuce seedlings (L. sativa) in a 120-h static test. The responses measured include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components that adversely affect the physiological and biochemical functions of the test organisms. This method should be performed by, or under the supervision of, professionals experienced in environmental toxicity testing.

Detection limits of the toxicity of a hazardous waste solution or pure substance are organism dependent.

### A.8.7.2 Summary of Method

Lettuce seeds (L. sativa) are exposed to different concentrations of hazardous waste solutions on wet filter paper for 120 h in the dark. Test results are based on the percent inhibition of seedling lettuce root elongation compared to controls.

### A.8.7.3 Definitions

For definitions of key terms, refer to the Glossary.

### A.8.7.4 Interferences

Toxic substances may be introduced by contaminants in water, glassware, sample hardware, artificial soil, and testing equipment (see Section A.4, Facilities and Equipment).

Improper hazardous waste sampling and handling may adversely affect test results.

Pathogenic organisms in test materials may affect test organism survival and growth, and also confound test results.

### A.8.7.5 Safety

For a discussion on safety, see Section A.2, Health and Safety.

### A.8.7.6 Apparatus and Equipment

1. Wire mesh screens for sizing seeds (fractions of an inch): 1/6 x 1/28, 1/6 x 1/30, 1/6 x 1/32, 1/6 x 1/3, A.T. Ferrell and Company, Saginaw, MI 48601 or Seedburo Equipment Company, Chicago, IL 60607.
2. Forceps.
3. pH meter.
4. Storage bottles for the hazardous waste solutions (see Section A.7, Hazardous Waste Sampling and Handling).
5. 33-gal, black plastic garbage bags.
6. Metric ruler.

7. Whatman filter paper, grade 3, 9-cm.

8. An illuminated magnifier.

9. A glass plate -- for measuring root lengths.

10. Sample containers -- for sample shipment and storage (see Section A.7, Hazardous Waste Sampling and Handling).

11. Controlled environmental chamber capable of maintaining a uniform temperature of 24 ± 2°C.

12. Water purification system -- Millipore Milli-Q or equivalents.

13. Balance, top loading -- capable of weighing soil samples to 0.1 g.

14. Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus samples.

15. Test chambers -- glass petri dishes, 100-mm wide by 15-mm with covers.

16. Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10- to 1000-mL.

17. Volumetric pipets -- Class A, 1- to 100-mL.

18. Serological pipets -- 1- to 10-mL, graduated.

19. Pipet bulbs and fillers -- Propipet® or equivalent.

20. Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

21. pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Portable, field-grade instruments are acceptable.

22. National Bureau of Standards certified thermometer (see EPA Method 170.1, EPA 1979b).

A.8.7.7 <u>Reagents and Consumable Materials</u>

1. Reagent water -- defined as activated-carbon-filtered, distilled or deionized water that does not contain substances toxic to the test organisms. A water purification system may be used to generate reagent water (see number 12 above).

2. Aqueous hazardous waste sample or a hazardous waste elutriate (see Section A.7, Hazardous Waste Sampling and Handling).

3. Dilution water -- Deionized water.

4. Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, EPA 1979b).

5. pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see EPA Method 150.1, EPA 1979b).

6. Membranes and filling solutions for the dissolved oxygen probe (see EPA Method 360.1, EPA 1979b), or reagents for modified Winkler analysis.

7. Laboratory quality assurance samples and standards for the above methods.

8. Reference toxicant (see Section A.3, Quality Assurance).

9. Test organisms -- lettuce (butter crunch variety), Lactuca sativa L. The seeds used in this test are available from commercial seed companies. Seed from one lot should be purchased in amounts adequate for one year's testing. Information on seed lot, year, or growing season in which they are collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicide, repellents, etc.) seeds are acceptable for use in this toxicity test.

**A.8.7.8 Sample Collection, Preservation, and Storage**

For a discussion on collecting, preserving, and storing samples, see Section A.7, Hazard Waste Sampling and Handling.

**A.8.7.9 Calibration and Standardization**

For a discussion on calibration and standardization, see Section A.3, Quality Assurance.

**A.8.7.10 Quality Control**

For a discussion on quality control, see Section A.3, Quality Assurance.

**A.8.7.11 Procedures**

**A.8.7.11.1 Test Solutions--**

One of two dilution factors, 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1%, using only five concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but, because of the wide interval between test concentrations, provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5. A dilution factor of 0.5 is generally preferred.

Twenty milliliters of solution is required for each test concentration. The minimum volume of hazardous waste solution required to run the test with three replicates per test concentration is about 240 mL. Prepare enough test solution (approximately 500 mL) for the highest and lowest concentrations to provide 300 mL additional volume for routine chemical analyses. (See Section A.8.7.11.4)

**A.8.7.11.2 Start of the Test--**

Sample preparation should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of sample toxicity is not known, the maximum holding time should not exceed 36 h. Just prior to testing, the temperature of the test solutions should be adjusted to 24 ± 2°C.

After purchase, size grading of seeds is carried out on the entire seed lot. Small samples, 100-150 grams, are sized at a time.

In addition, the seed lot must be inspected. Trash, empty hulls, and damaged seeds are removed.

Nest the four wire mesh sizing screens, with the one containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan is used to collect the fraction that passes through all screens.

Pour the seeds onto the top screen, then shake the whole set of nested screens (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are set aside and saved. This procedure is repeated until all the seed in the lot is sized. The size class containing the most seed is selected and used for the duration of the tests. The seeds in each size class are divided into small lots, placed in separate envelopes or sacks, and stored in airtight, waterproof containers in a refrigerator at 4°C.

Place a sheet of Whatman No. 3 filter paper in each of three replicate 100-mm plastic petri dishes. Prepare 20 mL of each test concentration using deionized water to dilute the hazardous waste solutions to the appropriate test concentrations.

Each replicate petri dish containing filter paper receives 4 mL of test solution. (The remaining 8 mL may be saved for chemical analysis.) Place five seeds, equally spaced, in a circle on the filter paper, equidistant from the edge to the center. Place the lid on each petri dish.

Set the petri dishes in layers in a black 33-gal plastic garbage bag lining a cardboard box. (Randomize the position of test containers at the beginning of the test.) Place moist laboratory paper towels between layers to keep the humidity level elevated and to prevent drying of the filter paper. Tape the garbage bag and close the box to seal the system. The box is placed in a controlled environment chamber and incubated for 120 h at 24 ± 2°C.

**A.8.7.11.3 Light, Photoperiod, and Temperature--**

Incubate at 24 ± 2°C in the dark.

**A.8.7.11.4 Routine Chemical and Physical Analyses--**

At a minimum, the following measurements are made:

- The pH, alkalinity, and hardness of the test solutions should be measured at the beginning of the test.
- Temperature is measured at the beginning of each 24-h exposure period.

**A.8.7.11.5 Observations During the Test--**

No observations are taken during the test.

**A.8.7.11.6 Termination of the Test--**

Measurement of root length is made at 120 h from the start of dark incubation. It is important to measure each plate as nearly as possible to 120 h (not to exceed ± 30 min.).

Remove the seeds from the filter paper and place on the glass work surface. Measure the distance from the transition point between the hypocotyl and root to the tip of the root. At the transition between the hypocotyl and the primary root, the axis may be slightly swollen, contain a slight crook, or change noticeably in size. The lengths of all roots are measured to the nearest millimeter and entered on the data sheet. For additional descriptions and photographs helpful in making root measurements, see USDA (1952) and Wellington (1961).

**A.8.7.11.7 Acceptability of Test Results--**

For the test results to be acceptable, mean germination in the controls must be at least 90%.

**A.8.7.11.8 Summary of Test Conditions--**

A summary of test conditions is listed in Table A-11.

**A.8.7.12 Calculations**

The effect measured during the toxicity tests using lettuce (L. sativa) root elongation is percent inhibition of lettuce root elongation compared to controls.

See Section 2 in the main text for data analysis methods.

Report the EC50 and its 95% confidence limits. The EC50 is an estimate of the median effective concentration.

**A.8.7.13 Precision and Accuracy**

Section A.3, Quality Assurance, describes test precision; the accuracy of toxicity tests cannot be determined.

**TABLE A-11. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR LETTUCE (L. SATIVA) ROOT ELONGATION TEST**

| | | |
|---|---|---|
| 1. | Test type: | Static |
| 2. | Temperature (°C): | 24 ± 2°C |
| 3. | Light quality: | Dark |
| 4. | Light intensity: | Dark |
| 5. | Photoperiod: | Dark |
| 6. | Test vessel type and size: | Glass petri dishes, 100-mm wide by 15-mm high |
| 7. | Test solution volume: | 4 mL |
| 8. | Test Solution pH: | $\geq$ 4 but $\leq$ 10 |
| 9. | Dilution water: | Deionized water |
| 10. | Renewal of test materials: | None |
| 11. | Age of test organisms: | Seeds |
| 12. | Number of test organisms per chamber: | 5 |
| 13. | Number of replicate chambers per dilution: | 3 |
| 14. | Dilution factor: | 0.5 |
| 15. | Test duration: | 120 h |
| 16. | Effect measured: | Root elongation |

* If pH is outside this range, results may reflect pH toxicity. Adjustments of pH to 4.0 or 10.0 may result in altered toxicity of other constituents. See Sec 1.4 and A 7.7.

## SECTION A.9

## REPORT PREPARATION

The general format and content recommended for reports follows.

1. INTRODUCTION

    1.1 Sample number
    1.2 Toxicity testing objectives or requirements
    1.3 Hazardous waste site location
    1.4 Contractor (if toxicity testing is contracted)
        1.4.1 Name of firm
        1.4.2 Phone number
        1.4.3 Address

2. SITE CHARACTERISTICS

    2.1 Types of hazardous wastes known to be present
    2.2 Geology
    2.3 Hydrology
    2.4 Description of waste treatment
    2.5 Volume of waste present

3. SOURCE OF HAZARDOUS WASTE, ELUTRIATE, DILUTION WATER, AND ARTIFICIAL SOIL

    3.1 Samples
        3.1.1 Sampling point
        3.1.2 Collection dates and times
        3.1.3 Sample collection method
        3.1.4 Physical and chemical data
    3.2 Elutriate Preparation
    3.3 Dilution Water
        3.3.1 Source
        3.3.2 Collection date and time
        3.3.3 Pretreatment
        3.3.4 Physical and chemical characteristics
    3.4 Artificial Soil
        3.4.1 Composition
        3.4.2 Pretreatment
        3.4.3 Physical and chemical characteristics

4. TEST METHODS

    4.1 Toxicity test method used
    4.2 Endpoint(s) of test
    4.3 Deviations from reference method, if any, and the reason(s)
    4.4 Date and time test started
    4.5 Date and time test terminated
    4.6 Type of test chambers
    4.7 Volume of solution or hazardous waste used/chamber
    4.8 Number of organisms/test chamber
    4.9 Number of replicate test chambers/treatment

4.10 Acclimation of test organisms (mean and range)
4.11 Test temperature (mean and range)

5. TEST ORGANISMS

5.1 Scientific name (and variety, if any)
5.2 Age
5.3 Life stage
5.4 Mean length and weight (where applicable)
5.5 Source
5.6 Diseases and treatment (where applicable)

6. QUALITY ASSURANCE

6.1 Standard toxicant used and source
6.2 Date and time of most recent test
6.3 Dilution water used in test
6.4 Results (LC50 or EC50 and confidence limits)
6.5 Physical and chemical methods used

7. RESULTS

7.1 Provide raw biological data in tabular form, including daily records of affected organisms in each concentration (including controls)
7.2 Provide table of LC50s or EC50s
7.3 Indicate statistical methods to calculate endpoints
7.4 Provide summary table of physical and chemical data
7.5 Tabulate QA data

## SECTION A.10

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